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FINE NEEDLE CYTOLOGIC BIOPSY IN DISEASES OF THE SALIVARY GLANDS

By

THORBJORN BERGE and NILS SÖDERSTRÖM

Received 13 xi 62

In the diagnosis of salivary gland disease biopsy is often desirable, but resorted to only reluctantly because of cosmetic considerations, risks of nerve lesions, salivary fistulas, etc Such contra indications may be avoided using *fine needle biopsy for cytologic* diagnosis Infor- ... often less con ... contri- ... and our *own experiences* with the method sufficiently encouraging to merit a short report, this all the more as the literature on the subject is still very scanty (*Lopes Cardozo* 1954 *Paseyro* 1956, *Söderström* 1958)

METHOD

For the puncture we have used ordinary hypodermic needles with an outer dia ... ls were ... tinuous ... tissue ... ad and ... routine

MATERIAL

Our clinical material consists *of 90 patients* with salivary swellings of different type Most of the patients were treated in the *out patient* services of the departments of otiatrics internal medicine and general surgery of this hospital The hospital has no specialized *tumor clinic* and the representation of different types of salivary gland diseases in this *material can be* assumed to reflect their relative frequencies in general practice with the exception that epidemic parotitis is probably not represented

CYTOLOGIC FINDINGS

The main constituent of most punctates is peripheral blood A gain of clear salivary fluid indicates the presence of cystic cavities In the absence of tumor or inflammatory reactions the smears are usually very poor in tissue cells The typical finding in such smears is a small

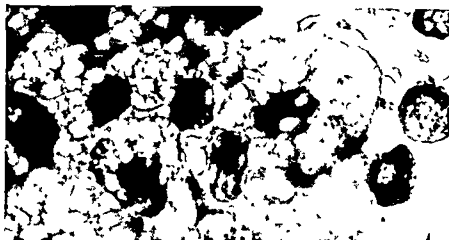


Fig 1
Detail of a cluster of large glandular cells from a salivary gland (submaxillary) The cytoplasmic vacuoles somewhat larger than usual (× 1000)

number of *large glandular cells* with a densely vacuolated "foamy" cytoplasma, sometimes containing small granules staining dark violet (Fig 1, 2) Such cells often occur in groups, to be recognized as remnants of glandular acini, which may be united with fragments of efferent ducts, built up by *small, basophilic, cuboid cells* At the point of junction between efferent ducts and acini somewhat larger basophilic cells sometimes seem to represent *transitional forms* between duct epithelium and gland cells (Fig 3) When occurring isolated the basophilic cells have a very nonspecific appearance and may be mistaken for lymphocytes

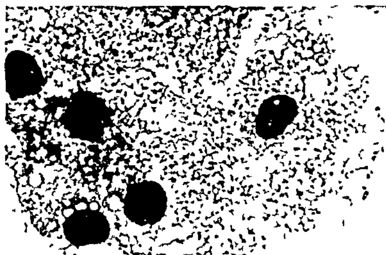


Fig 2
Large glandular cells from the parotid gland Detail of a burst acinus The typical granulation (paravacuolar granulation) is not always as distinct as here (× 800)

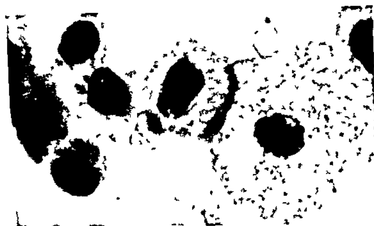


Fig 3
Cuboid duct cells (left) transitional cell (centre) and large glandular cell (right) (Parotid gland punctate × 1000)

The gland cells may be extremely scarce but their appearance is sufficiently characteristic to permit the *identification* of the structure punctured as a salivary gland This simple identification is often a performance of clinical importance—salivary gland swellings are often mistaken for being lymphomas etc before the puncture

Tumor punctates are usually rich in cells A very typical picture is offered by *pleomorphic adenomas* which are dominated by a rather uniform population of mediumsized basophilic cells, with dense nuclei, sometimes similar to the transitional cells described above (Fig 4 5), in some cases they may be similar to plasma cells In punctates from female patients the nuclei often contain a distinct sex chromatine in

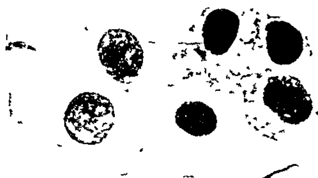


Fig 4
Tumor cells from a case of pleomorphic adenoma (× 1000)



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Detail of a cluster of large glandular cells from a salivary gland (submaxillary) The cytoplasmic vacuoles somewhat larger than usual (× 1000)

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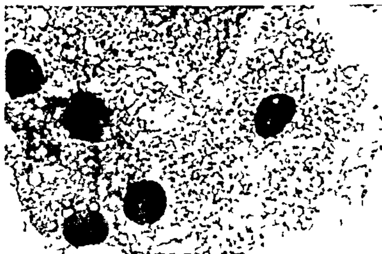
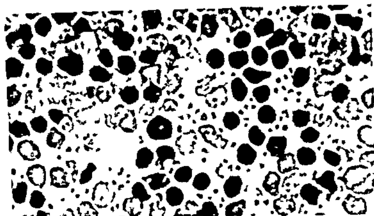


Fig 2

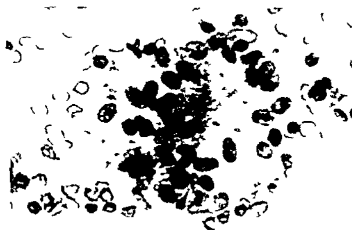
Large glandular cells from the parotid gland Detail of a burst acinus The typical granulation (paravacuolar granulation) is not always as distinct as here (× 800)



F g 7
Deta l of a parotid gland punctate fr m a case of Mikulicz disease The smear had the chara ter of a lymph node punctate dominated by lymphocytes prolymphocytes and lymphoblasts

In some cases unusually moderate degree of nuclear anisomorphism may indicate an increased tendency to proliferation (and thus to re currence after surgical removal) There was in our material a good agreement in this respect between the impression from the cytologic picture and the subsequent pathological anatomical statement based on the histology of the tumor

An overt *cancer* cytology represented by poorly differentiated and grossly polymorphic cells was present only in a few of our cases The



F g 8
C ant cell f the Langl ans t pe fr m a case f sarco dosis (I arot d gland punctate × ?00)

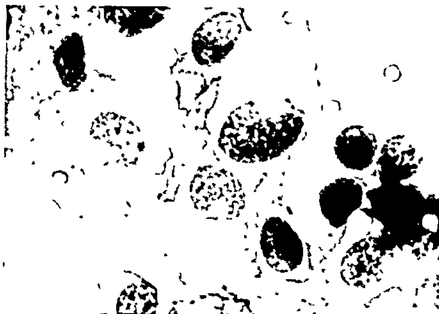


Fig 5
Tumor cells from a case of pleomorphic adenoma A distinct sex chromatine in 2 of the cells (× 1000)

marginal position The tumor cells usually occur together with mucoid substance staining brightly red, sometimes covering large areas of the smears, sometimes seen only as discrete stellate deposits (Fig 6) The attribute "pleomorphic" sounds little appropriate to the *cytologic* picture of this tumor, made up by a *monomorphous population* of epithelium cells, in which a conspicuous admixture of overt mesenchymal cells is seldom noted

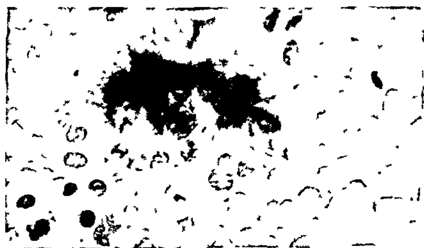


Fig 6
Pleomorphic adenoma Tumor cells together with mucoid substance (× 200)

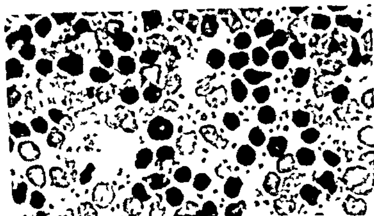


Fig 7
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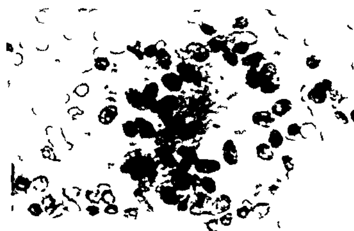


Fig 8
C ant cell f the Langlans t pe fr m a cas of sarcoidos s
(Par t d gland punctate × 200

rarer types of specific salivary gland tumors (*Foote & Frazell 1953*) were not represented in our material

A more or less conspicuous admixture of lymphocytes is often seen *and suggests the presence of chronic sialoadenitis* In extreme cases the smears may be dominated by a complete lymphoglandular cytology, comprising reticular stem cells and sinus endothelium cells mixed with glandular epithelium cells (lymphoglandular metaplasia) Lymphatic infiltrations of the salivary glands is an interesting chapter which is still far from settled to judge from the confusing nomenclature ("lympho epithelial lesion", Mikulicz disease, Sjogrens syndrome etc), (*Zeltergren* 1959) The cytologic picture gives the impression of different degrees of one and the same lesion and doesn't permit a detailed differentiation In addition it is wise to be cautious with the diagnostic conclusions· the punctates may be contaminated by lymphoglandular cells from separate lymph nodes lying close to the salivary gland (Fig 7)

Epitheloid cell granulomas are often clearly documented by the presence of epitheloid cells or giant cells of the Langhans' type The typical marginal position of the nuclei is often lost in the smears (Fig 8) In most cases this finding means a confirmation of a clinical suspicion of sarcoidosis (uveo-parotid fever, Heerfordt's syndrome), for the differential diagnosis against tuberculosis a staining for tubercle bacilli is, of course, necessary

The diagnosis of *cystic changes* is based on the macroscopic appearance of the punctate but also on the presence of large, free cyst phagocytes which may be similar to the glandular epithelium cells They *differ, however, by their character of "free" cells with distinct cell*-borders (isolated gland cells occurring in the smears usually have the indistinct, ragged cellborders of "tissue-bound" cells) Cystic changes usually represent the conditions labelled *sialectasis (Thackray* 1955, *Diamant* 1958, 1959), a heterogeneous condition which is often met with in this type of diagnostic work

The massive gain of neutrophilic leucocytes and tissue phagocytes met with in *acute sialoadenitis invariably* permits a reliable diagnosis of this condition

DIAGNOSTIC RESULTS

The cytologic conclusions were checked by histopathologic diagnosis (made by the pathologist of another hospital) in 28 cases only, reported in Table 1 In the remaining 62 cases a surgical intervention after the puncture was deemed unnecessary by the surgeon or refused by the patient It must not be forgotten that, normally, a conventional biopsy had probably not been resorted to as a routine diagnostic measure in most of these cases Some figures of Table 1 demand further comments

A definite cytologic diagnosis of *carcinoma* was made in 2 cases and confirmed by histopathology In 2 other cases the cytologic picture gave rise to a *suspicion* of malignant tumor, in one of these cases the eventual histologic diagnosis was cancer, in the other case not even the histologic diagnosis permitted more than a suspicion of cancer In a third case in which a proliferation of reticular stem cells was the basis for a cytologic *suspicion* of malignant reticulosis, the final histologic diagnosis was epitheloid cell granuloma

TABLE 1

Pathologicalanatomical diagnosis	Cytol diagnosis						
	Cancer	Malignant tumor suspected	Pleomorphic adenoma	Epitheloid cell granuloma	Chronic parotitis	Normal parotis	Inconclusive cytology
Cancer	2	1					
Malignant tumor suspected		1					
Pleomorphic adenoma (mixed tumour)			10				2
Epitheloid cell granuloma		1		5			
Chronic parotitis					1		1
Inconclusive histology						1	2
Normal parotis							1

The cytologic diagnosis of *pleomorphic adenoma* was made in 12 cases Histopathologic confirmation was obtained in 10 cases, in the remaining 2 cases the cytologic findings were typical, but no surgical intervention was made The diagnosis of pleomorphic adenoma was missed in 2 cases, in which the cytologic findings were inconclusive

In 5 cases the cytologic diagnosis of *epitheloid cell granuloma* was confirmed by histologic biopsy In another 2 cases epitheloid cells were found, but histologic control was not obtained and the clinical data did not permit a definite clinical diagnosis

The remaining cases, including the cases in which a histopathologic control was not obtained, represent miscellaneous conditions In some cases the punctates permitted more or less

rarer types of specific salivary gland tumors (*Foote & Frazell 1953*) were not represented in our material

A more or less conspicuous admixture of lymphocytes is often seen and suggests the presence of *chronic sialoadenitis* In extreme cases the smears may be dominated by a complete lymphoglandular cytology, comprising reticular stem cells and sinus endothelium cells mixed with glandular epithelium cells (lymphoglandular metaplasia) Lymphatic infiltrations of the salivary glands is an interesting chapter which is still far from settled to judge from the confusing nomenclature ("lympho-epithelial lesion", Mikulicz disease, Sjogrens syndrome etc), (*Zeltergren* 1959) The cytologic picture gives the impression of different degrees of one and the same lesion and doesn't permit a detailed differentiation In addition it is wise to be cautious with the diagnostic conclusions the punctates may be contaminated by lymphoglandular cells from separate lymph nodes lying close to the salivary gland (*Fig 7*)

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DISCUSSION

The main value of fine-needle biopsy in the field of salivary gland diseases lies in the fact that it permits a reasonably accurate diagnosis of some specific lesions (*e g* cancer, pleomorphic adenoma, sarcoidosis) as a result of an extremely simple and innocuous intervention Appropriate *therapeutic* measures may be based on such a positive cytologic diagnosis alone

The finding of cystic changes or lymphocytic infiltration of the gland tissue, is, of course, much less informative, confronted with other features of the clinical picture even such findings may, however, contribute in a valuable way to the diagnosis It is important to realize, however, that *information of this type is not to compare with a pathological-anatomical diagnosis* Only positive diagnostic findings are really conclusive, negative findings don't exclude the presence of specific lesions The limitations of the cytologic diagnosis must be clearly pointed out to the clinician To get the best out of the method a close personal contact between clinician and cytologist is desirable, if possible the cytologist should make the punctures himself If the gain of cells was insufficient in a first punctate he should not hesitate to secure a better material in a second puncture, in our experience no patient ever objected against a second puncture of this type

The clinical picture and the subsequent course of the disease generally confirmed the practical value also of less specific types of cytodiagnostic conclusions In spite of the fact that they often had to be formulated in cautious, not to say vague terms, they often proved to offer important information as a complement to other clinical findings There are still many obscure points in the pathology of the salivary glands, also the histologic diagnosis is difficult and often of restricted value in the clinical work It may be that information gained by cytologic biopsy is often very fragmentary, but if this is duely realized, such fragments of information may be valuable building-stones in the final diagnosis

SUMMARY

The authors report personal experiences with fine-needle cytologic biopsy in diseases of the salivary glands The method is stated to be technically simple and free of inconveniences and risks for the patient It permits an accurate positive diagnosis of specific lesions such as pleomorphic adenoma, cancer and epitheloid cell granulomas in other cases a peremptory diagnostic conclusion is often not possible, but the information gained may nevertheless be of decisive clinical value (lymphocytic infiltration, cystic changes etc) As a whole the method must *be regarded as a diagnostic adjunct of considerable value*

female animals These were each given 0 1 ml of the tumour ascites intraperitoneally (tumour cell count 1 960 000 mm c, tumour blood content—3 per cent)

Six days after the injection of the tumour all the mice were killed One ml of the tumour ascites was centrifuged in a Wintrobe tube to obtain the PCV of the erythrocytes (*Hartveit* 1961) and the cell free supernatant ascitic fluid This supernatant was then added to the original tumour as in the above experiment and films were made in the same way The films were numbered 1-5 according to the amount of excess ascitic fluid used (Table 1) The end point of the reaction was recorded as the number of the tube in which all the tumour cells were either swollen or pyknotic, *i e* at which no healthy cells remained

TABLE 1

The Proportions of Whole Ehrlich Ascites Carcinoma in Cell Free Ascitic Fluid from the Same Mouse Used in the in vitro Tests

Tube number	Whole tumour, drops	Ascitic fluid drops
1	1	0
2	2	1
3	1	1
4	1	2
5	1	4

RESULTS

Experiment I Figure 1 shows the result of adding different amounts of ascitic fluid from the same tumour to the tumour cells (1a). The addition of small amounts of excess fluid resulted in the production of large injured cells (1b), greater amounts produced pyknosis (1c) and still greater amounts clumping (1d). The pyknotic cells were seen to be joined by fine bridges (Fig 2a) These bridges were also seen on dark ground illumination of the wet preparation (2b). These findings were constant in all the mice investigated

TABLE 2

The Me... Bl... [illegible] ...he Amount of Excess Ascitic ...Cells—Expressed as the Mean ... (SE) of the Sex Difference

	Series	No of mice	Mean	SD	SE	t	P
Blood content (%)	♂	14*	2 5	1 9	0 88	1 46	0 2 > P > 0 1
	♀	14*	1 2	1 6			
	♂ + ♀	28	1 8	1 9			
Tube number	♂	14*	1 7	0 9	0 33	1 52	0 2 > P > 0 1
	♀	14*	2 2	0 9			
	♂ + ♀	28	2 0	0 9			

* Tumour unsuitable for investigation in one mouse

Experiment II Table 2 shows the mean blood content of the tumours, *i e* the PCV of the erythrocytes (with the standard deviation, SD) and

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THE IN VITRO DEMONSTRATION OF A CYTOTOXIC FACTOR IN EHRLICH'S ASCITES CARCINOMA

By

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Received 14.viii.62

In a previous experiment (*Hartveit* 1962) the author found that the number of injured cells present in untreated Ehrlich ascites carcinoma at 6 days increases with the blood content of the tumour. When the blood content is particularly high the number is less than would be expected from the regression line. This finding, which has not been stressed previously, prompted the re-examination of the films from the tumours in the previous experiment. It was found that in the cases in which the number of injured cells was lower than expected many pyknotic cells were also present, while there were few in tumours with a low blood content.

In view of these findings, which suggest that there is a factor injurious to the tumour cells—a cytotoxic factor—in the ascitic fluid, it was decided to see if an excess of fluid over that present in vivo would increase the amount of cell damage produced (experiment I), and if this were so to see whether the different fluids varied in their ability to cause cell damage, and whether this ability was related to the tumour blood content (experiment II).

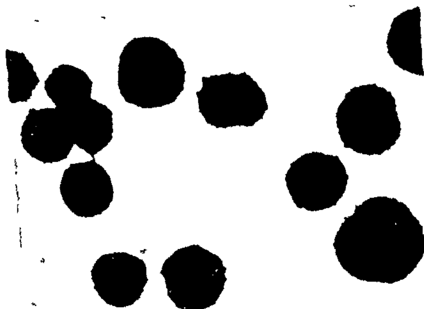
MATERIAL AND METHODS

The mice and the Ehrlich ascites carcinoma used were similar to those used in previous experiments (Hartveit 1961), the tumour now being in its 132nd transplant generation.

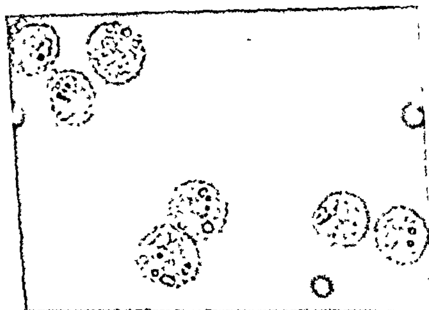
Experimental Procedure

Experiment I. One mouse provided the tumour for the experimental group which consisted of 5 male and 5 female animals that were each given 0.1 ml of the tumour ascites intraperitoneally (tumour cell count 1 370 000/mm c., tumour blood content —2 per cent). After 6 days the mice were killed and the tumour ascites removed. Half of the tumour ascites from each mouse was centrifuged to obtain the cell free ascitic fluid. This supernatant was then added to the other half of the tumour ascites using Pasteur pipettes in the proportions shown in Table 1. Films were made as described previously (Hartveit 1962) from the original tumours and from all dilutions.

Experiment II. (Based on the results of experiment I, vide infra). One mouse provided the tumour for this experimental group which consisted of 15 male and 15



a Leishman's stain × 1400



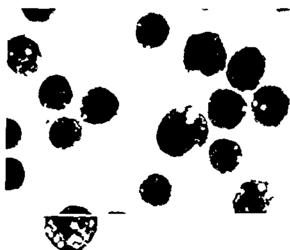
b Unstained wet preparation, dark ground illumination, × 1100

Fig 2

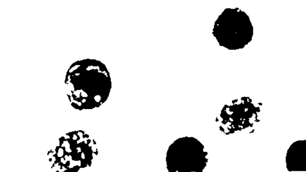
Bridge formation between pyknotic Ehrlich ascites carcinoma cells following the addition of an *excess* of cell free ascitic fluid from the same tumour to the unwashed cells

Fig 1

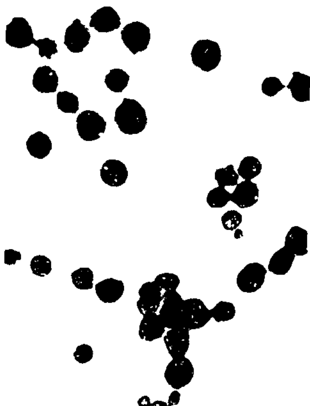
The effect of an excess of ascitic fluid from the same tumour on Ehrlich ascites *carcinoma cells*
Leishman's stain × 720 (reduced to approx × 580 in print)



a Whole tumour
Note normal cell size



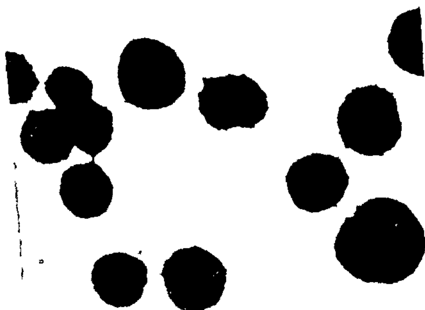
b 1 drop of tumour plus 1 drop of ascitic fluid Note swollen *cells and loss of cytoplasm*



c 1 drop of tumour plus 2 drops of ascitic fluid
Note pyknosis



d 1 drop of tumour plus 4 drops of ascitic fluid
Note pyknosis and clumping



a Leishman's stain × 1400

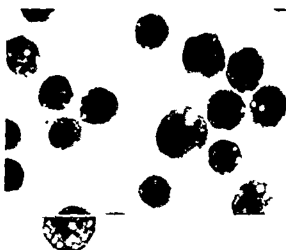
b Unstained wet preparation dark ground illumination × 1100

Fig 2

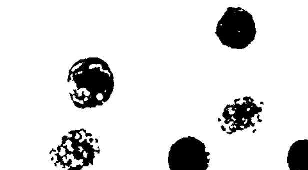
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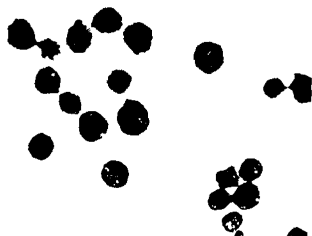
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Leishman's stain × 720
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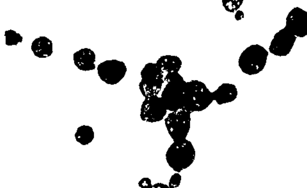
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c 1 drop of tumour plus 2 drops of ascitic fluid
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damage the tumour cells, and that the amount of damage varied from tumour to tumour Experiment I was therefore designed to see if the addition of ascitic fluid in excess of that found in vivo would increase the cell damage The results show that it did, small amounts producing the type of injury, seen in vivo that has been shown to be related to the tumour blood content (*Hartveit* 1962), larger amounts producing pyknosis and clumping of the tumour cells The formation of bridges between the pyknotic cells preceded clumping As these bridges were seen in the wet preparations they are not fixation artefacts The phenomenon is not purely mechanical as it occurred with different proportions of cells and ascitic fluid in different tumours (vide infra)

Direct reference to bridges of this type between tumour cells has not been found, but such a bridge has been clearly demonstrated in a photograph in *Fitch's* work (1962) on this tumour This photograph (*Fitch's* Fig 2A) shows the result of treating unwashed tumour cells with specific fluorescent labelled anti tumour globulin absorbed with mouse liver powder Bridges have also been shown to be present between erythrocytes when they have been agglutinated by specific antibody (*Stratton & Renton* 1958) Thus this finding adds further support to the idea (*Hartveit* 1962) that an immunological reaction is responsible for the cell damage

Experiment II was designed to see if the amount of this factor varies in the different ascitic fluids, and whether it is *related to the tumour* [illegible]

[illegible] to injure the cells of the tumour from which they were taken It was also clearly shown that this ability is related to the tumour blood content—a fluid from a tumour with a high blood content being more active than one from a non haemorrhagic tumour The considerable scatter in the results in Figure 3 may well be due to the experimental error inherent in the use of Pasteur pipettes as the drop size was not uniform different pipettes being used for tumour and ascitic fluid

The results of these experiments provide further evidence of the existence of a cytotoxic factor in untreated Ehrlich ascites carcinoma, and suggest that this *factor is related to the* immune reaction to the homografted tumour that is *reflected* in the tumour blood content (*Hartveit* 1961) There are two possible explanations of the findings The cytotoxic factor *may* be present in the [illegible] fluid that is added [illegible] [illegible] ctor in relation to [illegible] that the factor is [illegible] the unwashed cells and that an additional factor, present in the ascitic fluid, is needed in excess to trigger off the reaction In this connection it is of note that the interaction of Ehrlich ascites carcinoma cells and immune antiserum from a foreign host has been shown to take place only when complement has been added to the sy-

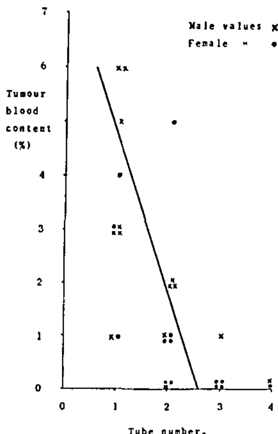


Fig 3

Regression line and scatter diagram of the relationship between the tumour blood content and the amount of excess tumour ascitic fluid needed to injure all the tumour cells—expressed as the tube number (see text), for the total series

the mean amount of excess ascitic fluid needed to produce changes in all the tumour cells (SD) for the male, female and total series The standard error (SE) of the difference between the male and female means is also given, with the t and P values

Table 2 shows that the sex difference was not significant for either factor Therefore the total series was used for the regression line (Fig 3) of the relationship between the tumour blood content (x) and the amount of excess ascitic fluid needed to produce changes in all the tumour cells (y), ($y = 2\ 58 - 0\ 33x$) The corresponding correlation coefficient, r, was found to be 0 6934 ($t = 3\ 602$) and as such was highly significant ($0\ 01 > P > 0\ 001$) ($r♂ = -0\ 6766$, $r♀ = -0\ 6517$)

DISCUSSION

The hypothesis on which the present experiments are based is that there is a cytotoxic factor in the ascitic fluid surrounding the Ehrlich ascites carcinoma cells A previous experiment (*Hartveit* 1962) had suggested that in some cases enough of this factor is present in vivo to

damage the tumour cells, and that the amount of damage varied from tumour to tumour Experiment I was therefore designed to see if the addition of ascitic fluid in excess of that found in vivo would increase the cell damage The results show that it did, small amounts producing the type of injury, seen in vivo, that has been shown to be related to the tumour blood content (*Hartveit* 1962), larger amounts producing pyknosis and clumping of the tumour cells The formation of bridges between the pyknotic cells preceded clumping As these bridges were seen in the wet preparations they are not fixation artefacts The phenomenon is not purely mechanical as it occurred with different proportions of cells and ascitic fluid in different tumours (vide infra)

Direct reference to bridges of this type between tumour cells has not been found, but such a bridge has been clearly demonstrated in a photograph in *Fitch's* work (1962) on this tumour This photograph (*Fitch's* Fig 2A) shows the result of treating unwashed tumour cells with specific fluorescent labelled anti-tumour globulin absorbed with mouse liver powder Bridges have also been shown to be present between erythrocytes when they have been agglutinated by specific antibody (*Stratton & Renton* 1958) Thus this finding adds further support to the idea (*Hartveit* 1962) that an immunological reaction is responsible for the cell damage

Experiment II was designed to see if the amount of this factor varies in the different ascitic fluids, and whether it is related to the tumour blood content, as suggested from the findings in a previous experiment (*Hartveit* 1962) It was found that the different fluids did vary (Fig 3) in their ability to injure the cells of the tumour from which they were taken It was also clearly shown that this ability is related to the tumour blood content—a fluid from a tumour with a high blood content being more active than one from a non haemorrhagic tumour The considerable scatter in the results in Figure 3 may well be due to the experimental error inherent in the use of Pasteur pipettes, as the drop size was not uniform, different pipettes being used for tumour and ascitic fluid

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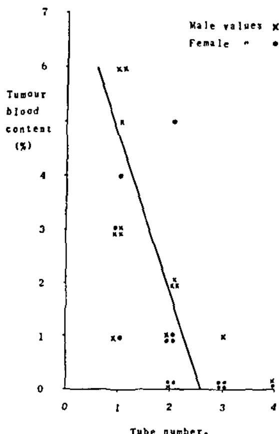


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stem (*Flax* 1956, *Wissler & Flax* 1957, *Fitch* 1962, *Stone Dzoga & Wissler* 1962) Similarly, with the Yoshida ascites sarcoma complement was needed to produce the in vitro changes in the tumour cells (*Lund* 1957) Further experiments on the mode of action of the cytotoxic factor in Ehrlich s ascites carcinoma are in progress

SUMMARY

The presence of a factor in untreated Ehrlich ascites carcinoma that is capable of damaging the tumour cells in vitro is demonstrated The morphology of the injured cells and the formation of bridges between the pyknotic cells suggest that an immunological reaction is involved This is also in accordance with the finding that the activity of the tumours in this respect is proportional to their blood content, a measure of the immune reaction of the host to the tumour homograft (*Hartveit* 1961)

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(HEAD PROFESSOR E WAALER M D)

THE VIABILITY OF PYKNOTIC EHRLICH ASCITES CARCINOMA CELLS

By

F HARTVEIT

Received 14 viii 62

In describing the stages in the death of an Ehrlich ascites carcinoma cell *King, Paulson, Puckett & Krebs* (1959) have shown that the cell bursts before it becomes pyknotic The findings in autolytic tumour support this view (*Hartveit* 1962b) The author has reported previously (*Hartveit* 1963) that Ehrlich ascites carcinoma cells treated with an excess of ascitic fluid from the same tumour become swollen, if the amount of excess fluid is low, and pyknotic if the amount is greater The latter cells are obviously damaged but may or may not be dead To settle this point the survival time of mice after the intraperitoneal injection of undiluted tumour, tumour diluted in physiological saline and tumour diluted with cell free tumour ascitic fluid was studied

MATERIAL AND METHODS

The mice and the Ehrlich ascites carcinoma used were similar to those used in previous experiments (*Hartveit* 1961) the tumour now being in its 130th transplant generation

Experimental procedure Five male mice that had received 0 1 ml [illegible]

[illegible] centrifuged and the supernatant ascitic fluid pooled

[illegible] male and 10 female mice were set up [illegible] peritoneal injection of 0 1 ml of the [illegible] the same amount of tumour ascites [illegible] ogical saline and group III the same [illegible] with pooled cell free tumour ascitic [illegible]

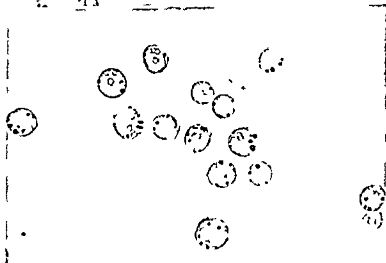
Investigations Films were made as described previously (Hartveit 1962 b) from the tumour injected in each group [illegible]

[illegible]

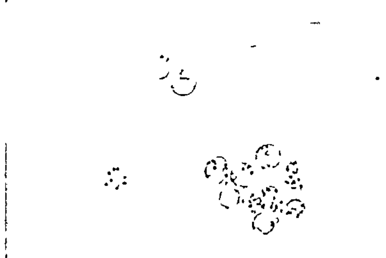
Fig. 1
The Ehrlich ascites carcinoma cells injected in the 3 groups
Dark ground illumination × 560 (reduced to approx × 450 in print)



a Group I
Note healthy cells



b Group II
Note slight shrinkage



c Group III
Note pyknosis and clumping

a Group I Note normal cell size

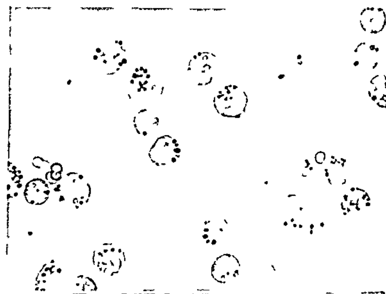


b Group III Note pyknosis and clumping

Fig 2

The Ehrlich ascites carcinoma cells injected in group I, undiluted, and in group III, diluted with ascitic fluid Leishman's stain, × 700

Fig 1
The Ehrlich ascites carcinoma cells injected in the 3 groups
Dark ground illumination × 560 (reduced to approx × 450 in print)



a Group I
Note healthy cells



b Group II
Note slight shrinkage



c Group III
Note pyknosis and clumping

TABLE 2

*The Survival Time ($\bar{x}$) in Days (SD) of the Mice in the 3 Groups (see Table 1 a)
The Sex Differences within the Groups with SE t and P Values Are Given
(10 Male and 10 Female Mice in each Group)*

Group	Series	$\bar{x}$	SDx	Sex diff $\bar{x}$	SE	t	P
I	♂	13 5	2 4	1 5	0 93	1 61	0 2 > P > 0 1
	♀	15 0	2 2				
II	♂	15 4	2 7	1 8	1 24	1 45	
	♀	13 6	2 8				
III	♂	14 4	1 9	2 3	1 08	2 12	0 05 > P > 0 02
	♀	16 7	2 8				

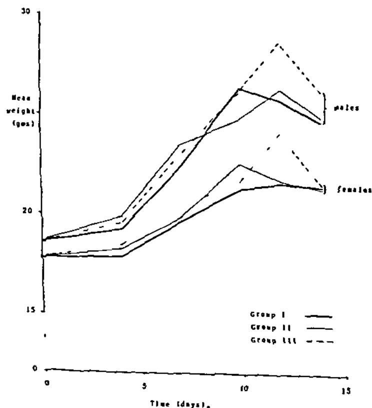


Fig 3

The mean weight of the mice in the different gr

RESULTS

Figure 1 shows wet preparations of the tumour injected in each of the experimental groups. The tumour cells in Figure 1a—group I, injected undiluted—appear healthy. Some of those in Figure 1b—group II, diluted with saline—are slightly shrunken. The cells in Figure 1c—group III, diluted with ascitic fluid—are pyknotic and show clumping. This pyknosis is further evident in Figure 2 which shows the undiluted cells (2a) and the cells diluted in ascitic fluid (2b). The pyknosis of the cells in the latter fluid was uniform, no healthy cells remaining. The effect of diluting with pooled ascitic fluids appeared identical to that previously seen on adding an excess of fluid from the same tumour (*Hartveit* 1963).

TABLE 1a

The Diameter (x) of the Ehrlich Ascites Carcinoma Cells in the 3 Groups I—Given Undiluted Ehrlich Ascites Carcinoma II—Tumour Diluted with Saline and III—Tumour Diluted with Ascitic Fluid as Measured from Photographs

Group	$\bar{x}$ (mm)	SD_x
I	9.16	1.41
II	8.38	1.17
III	6.56	0.73

TABLE 1b

The Difference in Cell Diameter (x) between the 3 Groups (see Table 1a) with the SE, t and P Values

Difference between groups	Diff in $\bar{x}$ (mm)	SE	t	P
I and II	0.78	0.41	1.77	$0.1 > P > 0.05$
II and III	1.82	0.69	2.64	$0.02 > P > 0.01$
I and III	2.60	0.39	6.72	$0.001 > P$

Table 1a gives the mean cell diameter, with standard deviation (SD) of the cells injected in each group, and shows that slight shrinkage occurred in cells diluted with saline and marked shrinkage in those diluted with ascitic fluid. Table 1b shows the differences in cell diameter between the groups, with the standard error (SE), t and P values. The amount of shrinkage in the saline diluted cells is not significant, while that of those diluted with ascitic fluid is highly so ($0.001 > P$).

Table 2 gives the survival time (SD) of the mice in all three groups and the sex differences within the groups, with SE, t and P values. The sex difference was only significant in group III ($0.05 > P > 0.02$).

TABLE 2

The Survival Time (x̄) in Days (SD) of the Mice in the 3 Groups (see Table 1a)
The Sex Differences within the Groups with SE, t and P Values Are Given
(10 Male and 10 Female Mice in each Group)

Group	Series	x̄	SDx	Sex diff x̄	SE	t	P
I	♂	13.5	2.4	1.5	0.93	1.61	0.2 > P > 0.1
	♀	15.0	2.2				
II	♂	15.4	2.7	1.8	1.24	1.45	
	♀	13.6	2.8				
III	♂	14.4	1.9	2.3	1.08	2.12	0.05 > P > 0.02
	♀	16.7	2.8				

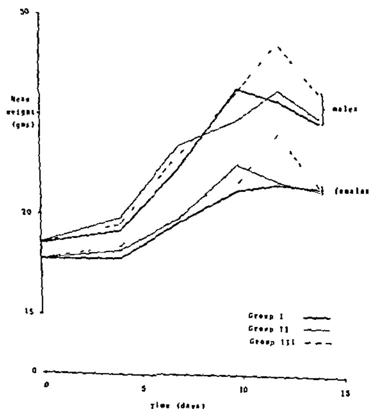


Fig 3

The mean weight of the mice in the different groups

Table 3 gives the difference in survival time between the groups, with SE, t and P values The difference between the females in groups II and III was *significant* (0 05 > P > 0 02)

TABLE 3

The Difference in Survival Time (x) in Days between the 3 groups (see Table 1 a) with the SE t and P Values (10 Male and 10 Female Mice in each Group)

Difference between groups	Series	Sex diff x	SE	t	P
I and II	♂	1 9	1 15	1 65	0 2 > P > 0 1
	♀	1 4	1 00	1 39	"
II and III	♂	1 0	0 62	1 61	
	♀	3 1	1 26	2 47	*0 05 > P > 0 02*
I and III	♂	0 9	0 97	0 93	0 4 > P > 0 3
	♀	1 7	1 05	1 62	0 2 > P > 0 1

On vital staining of the tumour cells in the three fluids injected none of the tumour cells took up the vital stain at 2 minutes, at 15 minutes or at 45 minutes, at which time all the injections had been completed After 2 hours 7 per cent of the cells in the group I fluid, 10 per cent in group II, and 50 per cent in the group III fluid took up the vital stain

Figure 3 shows the weights of the mice in the 3 groups related to time The differences between the groups are not statistically significant

DISCUSSION

The results of this experiment show that although morphological signs of injury can be seen in Ehrlich ascites carcinoma cells treated with an excess of tumour ascitic fluid the cells are not dead This is *in keeping with the results of vital staining but not in keeping with the* idea that the pyknotic tumour cells are cells that have burst *King, Paulson, Puckett & Krebs* (1959) have shown that damaged tumour cells swell, burst and subsequently become pyknotic and take up the vital stain The author found the same in cells undergoing non-specific autolysis (*Hartveit* 1962b) It has also been shown that when a small amount of excess ascitic fluid is added to the tumour cells swelling results, *but when a large* amount of fluid is added all the cells become pyknotic (*Hartveit* 1963), preliminary swelling has not been observed

This suggests that the injury inflicted by a great excess of the fluid differs from the primarily cytoplasmic injury that follows a smaller amount of the same fluid, in that the cell does not burst This idea is supported by the findings in the present experiment Vital staining showed that the tumour cells that *had been diluted with ascitic fluid*

did not take up the stain after 2 minutes—as they should have done had they been burst cells (*King Paulson Puckett & Krebs* 1959) After 2 hours half of them did so—in contrast to the controls—confirming that some damage was present

The survival time of the mice in the present experiment clearly indicates that the pyknotic tumour cells were not dead (Table 3—the difference in survival time between the untreated cells (group I) and those diluted with ascitic fluid (group III) was not significant The weight curves for the mice in the different groups (Fig 3) show that there was no preliminary lag in group III that could indicate that a lower dose of living cells had been compensated by an XYZ effect exerted by the dead cells present

The changes in cell diameter and morphology (Figs 1 and 2 and Table Ia) suggest that the cells in group III are damaged This is supported by the results of the vital staining and of the survival time experiments in the females (vide infra) It has been shown previously that female mice have greater natural resistance to Ehrlich s ascites carcinoma than males (*Hartveit* 1962a) This does not usually show up on intraperitoneal injection But in group III of this experiment the females survived significantly longer than the males (0 05 > P > 0 02) Table 2 This finding suggests that the cells were damaged so that they were more vulnerable than usual to the immune response of the host This is further supported by the finding (Table 3) that the females in group III also survived significantly longer than those in group II (0 05 > P > 0 02) The group II cells had been diluted in saline *i e* they had gone through the same mechanical process as those in group III but their vitality was not impaired in contrast to that of the cells diluted in ascitic fluid

It is of note that Ehrlich ascites carcinoma cells treated with heterologous immune gamma globulin show swelling Whether or not rupture of the cells takes place is debated (*Flax* 1956 *Green Barrow & Goldberg* 1959) but their viability is definitely reduced (*Lindner* 1960) The changes in such cells appear to be identical to those following a small amount of excess ascitic fluid and the reduced viability is in keeping with the finding that the cells with the latter changes show vital staining after about 10 minutes (*Hartveit* 1962b) in contrast to the pyknotic cells in the present experiment that needed two hours

It thus seems that the injury to the tumour cells following a great excess of ascitic fluid is reversible at least in the early stages while that following smaller amounts may not be This possibility is being investigated

SUMMARY

Ehrlich ascites carcinoma cells treated with an excess of tumour ascitic fluid become pyknotic These pyknotic cells are not dead but injured It is suggested that the pyknosis of the cells has not been pre

ceded by cell rupture with consequent irreversible cell damage. The experiment also presents further evidence that female mice show greater natural immunity to the Ehrlich ascites carcinoma than male mice.

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SEX DIFFERENCES IN THE INTRAPERITONEAL GROWTH OF EHRLICH'S ASCITES CARCINOMA

By

F HARTVEIT

Received 29 viii 62

Previous experiments have indicated that there is a difference in the reaction of male and female mice to the transplantation of Ehrlich's ascites carcinoma This difference does not appear to effect the survival time of the animals following intraperitoneal injection of the tumour (*Hartveit* 1961a) but it does show up on subcutaneous injection (*Hartveit* 1962a) It does not seem to be reflected in the blood content of the intraperitoneal tumour (*Hartveit* 1961a & b) but it does show up on the transplantation of pyknotic tumour cells (*Hartveit* 1963b) From these experiments it appears that female mice may possess more natural immunity to the homotransplant than males

The following experiment was designed to study the morphology of the Ehrlich ascites carcinoma cells at various times after transplantation to see if this would give any further information on the above mentioned sex difference

MATERIAL AND METHODS

The mice and the Ehrlich ascites carcinoma used were similar to those used in previous experiments (*Hartveit* 1961 a) the tumour now being in its 133rd transplant generation

Experimental pro[illegible]

a [illegible]

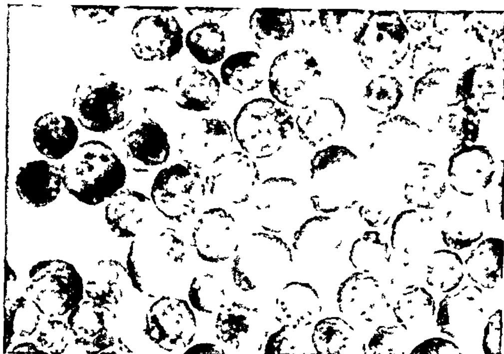
e [illegible]

[illegible] cell count 1 080 000 mm^3—tumour blood content—1 per cent)

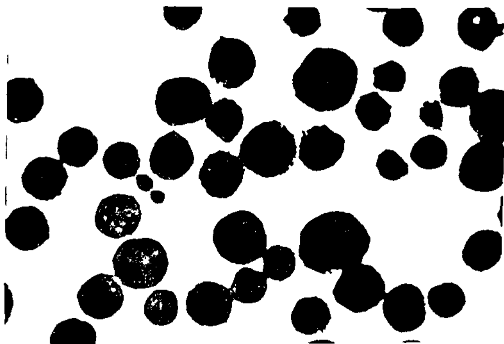
Serial biopsy specimens were taken from these mice at three day intervals by inserting a needle through the abdominal wall and collecting the drop of tumour ascites that formed when the needle was withdrawn Films were made [illegible] fluid and stained [illegible]
tumour cells was [illegible]
was counted as [illegible]
film and the resu[illegible]

RESULTS

Four main types of Ehrlich ascites carcinoma cells were seen These were the normal cells (Fig 1a), pyknotic cells (Fig 1b) and two types



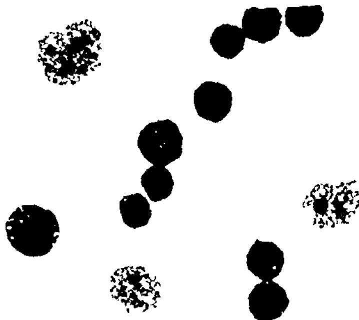
a Biopsy specimen of tumour 3 days after tranplantation Normal cells Note size shape and stainability



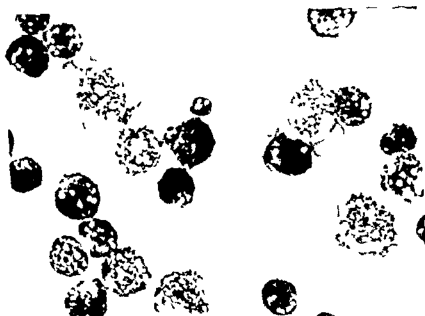
b Biopsy specimen of tumour from the same mouse [illegible] days after transplantation Note flattening of cell surface pyknosis increased stainability intercellular bridges and clumping

Fig 1

Changes in untreated Ehrlich ascites carcinoma cells following transplantation Leishman's stain × 700



a Type 1 from first biopsy showing pyknosis Note disintegration of cytoplasm and presence of nucleoli



b Type 2 from biopsy 9 days later Note simultaneous disruption of cytoplasm and nucleus with loss of nucleoli Also transition via lesser changes of same type from pyknotic to large cells

Fig 2

Large injured tumour cells in untreated Ehrlich ascites carcinoma. Leishman's stain × 700

of large injured tumour cell The first of these (type 1) was similar to those described previously (*Hartveit* 1962b) that showed swelling and primary cytoplasmic damage indicative of immunological type damage (*Fig* 2a) The second type of large injured tumour cell (*type 2*) was clearly different from the type 1 cells in that the nuclear and cytoplasmic damage was seen to occur simultaneously and transition stages between these and the pyknotic tumour cells were seen (Fig 2b)

Normal and type 1 tumour cells were present in early biopsies After a certain time, which varied from mouse to mouse, the majority of the tumour cells lost their previous spherical shape—their surface showed *flat planes*—and pyknosis occurred, with increased stainability Intercellular bridges were also seen between these pyknotic cells and clumping was also present (Figs 1a and 1b) A few type 1 cells were still present in the first biopsy showing pyknosis but after this time the tumour was made up of pyknotic and type 2 cells

Figure 3 shows the time of occurrence of pyknosis There was a marked sex difference Pyknosis tended to occur earlier in the tumours in female mice It was present in 6 of these at 3 days while none of the males had pyknotic tumours at this time This difference is statistically significant (0 001 > P)

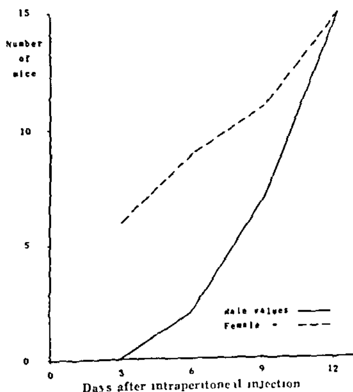


Fig 3

The time of occurrence of *in vivo* pyknosis in Ehrlich s ascites carcinoma (15 ♂ and 15 ♀ mice)

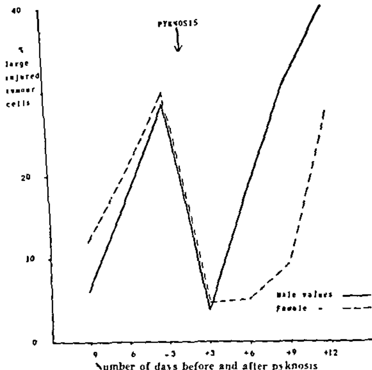


Fig 4

The mean percentage of large injured Ehrlich ascites carcinoma cells present in biopsies taken before and after *in vivo* pyknosis of the tumour (see Table 1)

TABLE 1

The Percentage of Large Injured Tumour Cells (Types 1 & 2) in Untreated Ehrlich Ascites Carcinoma Related to the Time of in vivo Pyknosis of the Tumour Cells (Data from 15 ♂ & 15 ♀ Mice)

		Days before pyknosis*			Days after pyknosis*			
		9	6	3	3	6	9	12
Large injured tumour cells		Type 1				Type 2		
Mean %	♂	6 6	17 2	28 1	4 0	17 6	31 5	39 0
	♀	12 0	20 2	29 9	4 6	5 0	9 0	20 7
S D	♂	7 8	10 0	6 2	2 1	13 3	7 9	7.0
	♀	6 5	7 9	8 1	2 4	2 9	11 0	17 5
Number of biopsies	♂	8	13	15	13	9	6	2
	♀	3 §	5 §	9	15	14	12	8

* Maximum time possible § Biopsy unsuccessful in one other mouse

Table 1 shows the mean percentage of large damaged tumour cells, of both types, present in the biopsies preceding and following the occurrence of pyknosis. The standard deviation of these values and the number of biopsies studied is given. The results are shown graphically in Fig. 4.

In both sexes the number of injured tumour cells—type 1—rose to a peak in the biopsy preceding pyknosis and fell sharply when pyknosis had occurred. This drop in the number of type 1 cells is highly significant in both sexes (0.001 > P). After this time a further sex difference is apparent. The number of type 2 cells rose more quickly in the males than in the females, the differences in the two biopsies following the first showing pyknosis (6 and 9) being significant (0.001 > P).

DISCUSSION

The results of serial biopsy in the present experiment in which the number of injured tumour cells was seen to increase with time, and to reach a peak which was followed by pyknosis of the cells, are reminiscent of the findings on adding an excess of ascitic fluid to the tumour cells (*Hartveit* 1963 c). In this previous experiment it was seen that when few large injured cells were present in vivo the addition of excess ascitic fluid increased their numbers and an even greater excess resulted in the pyknosis of the cells. The large injured cells produced were of the type seen in early biopsies in the present experiment—type 1 cells. The addition of even greater amounts of excess ascitic fluid did not give rise to type 2 cells as were seen in the later biopsies.

This indicates that although an increase in the amount of a cytotoxic factor may be responsible for the injury seen in type 1 cells it is unlikely to be the cause of the injury seen in the type 2 cells. This idea is under investigation and is supported by the morphology of the type 2 cells, in which the injury appears to effect both nucleus and cytoplasm simultaneously. In addition, as shown in Fig. 2b, the majority of the cells show minor changes of a similar nature, changes that are more like those seen on in vitro autolysis (*Hartveit* 1962b) than those due to immunological damage. It is suggested that these cell changes are probably due to anoxia, particularly as they were not seen until later biopsies when the amount of ascites was large and the host moribund.

A striking finding was that the biopsy with the greatest number of type 1 cells was invariably followed by a biopsy containing only a few injured cells, the rest being pyknotic. These pyknotic cells are undoubtedly viable. *Klein & Revesz* (1953) found no difference in the amount of inoculum needed to produce an ascitic tumour from tumours between 3 and 16 days after transplantation. This is supported by the author's own experience that a 10 day tumour, in which the cells are usually pyknotic, is as reliable for transplantation as a 6 day tumour in which the cells are of normal appearance. In addition tumour cells

of similar pyknotic appearance produced by adding an excess of ascitic fluid to the tumour ascites are viable (*Hartveit* 1963b).

It thus seems that after a critical percentage of injured tumour cells is present the remaining cells shrink and appear pyknotic. This critical percentage seems to be around 30 per cent and to be the same in both sexes. The day this peak is reached varies greatly (presumably due to phenotypic and genotypic variations in the mice) and here there is a marked sex difference, the peak and subsequent pyknosis occurring before the 3rd day in 6 of the females in contrast to the males (Fig. 2). This suggests that the immunological response occurs earlier in the females than in the males.

A further marked sex difference is that the number of large injured cells in the two biopsies following that showing pyknosis was lower in the females than in the males (Fig. 4). This is probably a reflection of the fact that pyknosis tended to occur earlier in the females and that the tumours were not so large by the time these biopsies were taken. Incidentally, this further supports the idea that the type 2 changes are the result of anoxia.

Unfortunately the cytological findings cannot be related to the survival time in this experiment as serial biopsy in itself may interfere with the survival time. Similarly the blood content of the tumour (*Hartveit* 1961b) cannot be measured reliably. However, the findings show that the greater immunity of female mice to the subcutaneous injection of Ehrlich's ascites carcinoma (*Hartveit* 1962a) is reflected in the morphology of the intraperitoneal tumour, in that the results of the immune response (i.e. increase in the number of injured cells and pyknosis) are seen earlier in female mice.

While the time of occurrence of the immune response is different in the two sexes the mechanism of the response itself appears to be the same. There are no significant differences in values obtained up to and including the first biopsy showing pyknosis, and it is probable that the time factor is also responsible for the differences after this time (vide supra).

SUMMARY

The morphological changes in Ehrlich ascites carcinoma cells were followed by serial biopsy. The number of large injured cells was found to increase with time until a critical percentage (30 per cent) was reached. Thereafter there were a few large injured tumour cells while the rest were pyknotic. Large injured tumour cells reappeared in later biopsies but were of different morphology from those preceding pyknosis. It seems likely that the injury in the latter cells is due to immunological damage while that in the former may well be the result of anoxia.

There was a marked sex difference in the mode of growth of the tumour in that pyknosis occurred earlier in the females. This supports

the author's previous findings (*Hartveit* 1962a and 1963a) that female mice have greater natural immunity to Ehrlich's ascites carcinoma than males

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WHITE BLOOD CELLS FROM ANIMALS WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS TESTED ON GLIA CELLS IN TISSUE CULTURE

By

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The experimental allergic encephalomyelitis (EAE) that can be induced in various species by immunization with brain or spinal cord material, together with Freund's complete adjuvant, is usually supposed to be caused by a delayed type of hypersensitivity. The lymph cells are generally regarded as mediators of the disease. Passive transfer with immune serum has failed but transfer with cell suspensions from lymph nodes of immunized rabbits, guinea pigs, and rats has been successful. Reviews on these problems are given by *Waksman* (1959) *and Chase* (1959), and more recent studies on cellular transfer of the disease are presented by, among others, *Paterson* (1960), *Stone* (1961), and *Åström & Waksman* (1962).

The importance of circulating serum antibodies, however, was stressed in recent studies by *Bornstein et al.* (1961, 1962) and the present authors (*1962 a*). It was shown that sera from animals with EAE damaged glia cells and myelin in tissue cultures prepared from neonatal rat brains. Further interest in this phenomenon was evoked by the observations (*Berg & Källén* 1961, 1962 b, *Bornstein* 1962) that similar factors were found in sera from patients with neurological diseases, among these multiple sclerosis. Among other things, the negative results of passive transfer of serum at EAE argue against a pathogenetic importance of serum factors.

In the present study, a toxic effect of white blood cells from rabbits with EAE on neonatal rat glia cells, cultured on reconstituted rat tail collagen, is described. *Goverts* (1960) showed that [illegible]

[illegible] by *Stuart* (1962). *Sharp & Burwell* (1962) extensively discussed this literature and also presented evidence for a growth arresting effect of sensitized lymph cells on fibroblastic growth from spleen tissue. In most of these studies, complement was

not added to the test systems *Klein & Sjogren* (1960) incubated *in vitro* tumour cells with lymph cells immunized against the tumour cells, and showed that a destruction of the tumour cells occurred No complement was present

Since, the preparation of the present paper, a very similar study has appeared, written by *Koprowski & Fernandes* (1962), describing a "contactual agglutination" of sensitized lymph node cells from EAE animals in brain tissue culture, accompanied by destruction of glial elements

MATERIAL AND METHODS

Rabbits and rats were used as experimental animals The rabbits were of mixed stock supplied commercially The rats belonged to the homozygous Amsterdam R strain

Immunization was performed with porcine spinal cord and Freund's complete adjuvant (Difco) The rabbits received injections into one foot pad of each of three legs the intradermal doses injected being 0 05 ml Rats received 5–6 dorsal intra dermal injections of each 0 05 0 1 ml 6 rabbits were injected with pig kidney tissue and Freund's complete adjuvant

At various times after immunization blood was collected from the rabbits usually by carotid bleeding The blood was allowed to flow freely into a test tube con taining 6 per cent dextran (Macrodex, Pharmacia) and a few drops of heparin The proportions of dextran and blood was roughly 1 3 The blood was left to sediment and the clear fluid containing most of the white blood cells and some red cells was removed after 15 45 minutes The cell suspension was centrifuged at appr 1000 r p m washed with a large amount of Parker 199 medium, spun down again and re-suspended in Parker 199 A cell count of 2 5 millions/ml was obtained This suspension was added to the cultures

In a few rabbits, the thoracic duct was cannulated This operation was performed in morphine urethane anaesthesia After removal of the first rib on the left side of the animal a thin plastic cannule was inserted into the duct and the lymph which flowed freely was collected in a test tube containing a small amount of heparin in Parker 199 The cells were spun down and washed as described for the blood cells and a suspension of 3–4 millions/ml was prepared in Parker 199 Practi cally all cells obtained in this way are lymphocytes

The rats were cannulated through the thoracic duct using the method described by *Saldeen & Linder* (1960) These cells too were washed and re suspended

Glia cultures, prepared from neonatal rats of a mixed stock were used as a test system The cultures were made with reconstituted rat tail collagen The explants were put on top of the collagen and left to dry for a few hours A fluid medium consisting of 50 per cent human adult serum 5 per cent chick embryo extract and 45 per cent Tyrode salt solution increased with glucose to produce a concentration of 500 mg% was then added Antibiotics were included Incubation at 37 5° Growth commenced after 3 4 days and in most cultures a mat of neuroglia cells was found after appr one week

Before the tests the cultures were rinsed with Parker 199 The cell suspension to be tested was then added It was left in contact with the culture for appr 24 hours and then washed off Parker 199 was added as a medium In control cultures glia cells survived for a long time in this medium Phase contrast photomicrograms were taken before addition of the cells and at regular intervals during the follow ing 48 hours

RESULTS

1 *Scoring of the Tests*

"O" Glia cells and mesenchymal parts of the cultures survived well A strong branching of the glia cell processes was noted

"+" A slight thinning out of the glia cell mat occurred with de-

F g 1A

F g 1B

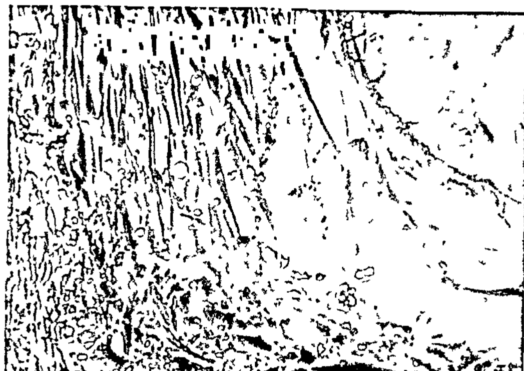


Fig 1 C

Fig 1 A–C

Phase contrast photomicrogram of a culture of neonatal rat brain A Culture before addition of sensitized white blood cells B The same microscopic field 48 hours later C Another microscopic field showing intact mesenchymal cells

generation of some glia cells but with good survival of most glia elements, which showed a normal appearance comparable to those in cultures scored "O"

"+ +" Heavy degeneration of glia cells occurred, leaving mesenchymal elements intact (Fig 1A, B, C) Numerous macrophages could usually be observed The rapidity of the degeneration varied in different experiments In most of these, a complete degeneration was found already at 24 hours, but in some not until 48 hours

Agglutination of lymph cells around glia elements, similar to that described by *Koprowski & Fernandes* (1962), was often seen In our opinion, however, the glia-cell degeneration is a more positive indication of the effect of the lymph cells

2 *Rabbit Experiments*

The results are summarized in Table 1

A) *Non-immunized animals* 10 animals were studied 6 were scored "O" both 24 and 48 hours after addition of the cells In the other 4, the cultures were scored "O" at 24 hours and "+" at 48 hours This faint cytopathic effect can thus occur in normal animals and is therefore without significance

TABLE 1

Rabbit Material

Animal number	Days after immunization	Clinical signs of EAE	Duration of clinical signs	Histological signs of EAE	Results of cell test: After 24 hrs	Results of cell test: After 48 hrs	Note
46	4	0		neg	0	0	
47*	5	0			0	0	
34	7	0		neg	0	0	
51	7	0		neg	0	+	Thoracic cells neg
46	8	0			0	+	Dead in EAF at 10 d
35	8	0		neg	++	++	
40	8	0		pos	0	0	
41*	8	0			++	++	Before immun neg
61	8	0		neg	++	++	Thoracic cells pos
36	9	0		neg	++	++	Before immun neg
52	9	0		neg	0	0	Thoracic cells neg
53	10	0		pos	+	++	
29	10	0		neg	++	++	
37	10	0		neg	0	++	Before immun neg
42	10	0		neg	0	0	Before immun neg
43*	10	0			++	++	Before immun neg
63	10	pos	1	pos	+	++	Thoracic cells pos
44	11	0		neg	+	0	Before immun neg
47§	12	pos	0	pos	++	++	
30	12	0		pos	0	++	
31	13	pos	1	pos	0	++	
41§	13	pos	3	pos	0	0	
32	14	pos	2		0	++	
33	15	pos	2		0	+	
48	19	pos	3	neg	0	0	
49	21	0		neg	++	++	
38	22	pos	1	pos	0	0	Before immun neg
39	26	0		pos	++	++	
64	29	pos	18	pos	0	0	
43§	37	0		neg	0	0	
45	37	pos	4	pos	0	0	

* First bleeding after immunization § Second bleeding after immunization

B) *Animals tested within one week after immunization* 4 animals belong to this group All were healthy, and the brains studied showed no signs of EAE In no case was a "+ +" effect seen

C) *Animals tested more than one week after immunization* 27 tests were made Of these, 15 were positive and 12 negative (cf Table 2)

TABLE 2

Summary of Test Results in Animals Bled more than 1 Week after Immunization

	Clinical signs of EAE: present	Clinical signs of EAE: absent	Total
Positive tests	4	11	15
Negative tests	6	6	12

Of the *15 positive tests,* only 4 were made on animals showing positive findings of clinical EAE Of the 11 without clinical signs of EAE, 3 had a positive histology The 4 animals with clinical signs of EAE had all shown symptoms for less than 2 days In the tests of three of these animals only a slow degeneration could be observed in the culture This was not noticeable 24 hours after addition of the cells

Of the *12 negative tests,* 6 animals showed symptoms of EAE and 6 did not Most of the former 6 had shown the clinical signs for more than 2 days One of the latter 6 had a positive histology Another had been positive in an earlier test The remaining 4 with a negative test in this group may not have been adequately immunized

As is well known, the timing of the clinical development of EAE is extremely difficult to assess Nevertheless, the trend of the investigations presented here suggests the possibility of finding a positive test in animals a few days before the appearance of clinical signs of EAE and during the first days of the disease

Three animals offer some points of special interest because repeated tests were performed on the same material

Animal No 47 This animal was negative 5 days after immunization, positive after 12 days, simultaneously with the development of clinical signs of EAE

Animal No 43 This animal was negative before immunization, positive 10 days after immunization, but never developed clinical signs of EAE After 37 days the test was again negative, and histology at this stage did not show any signs of EAE Nevertheless, it is not possible to preclude the presence of a slight EAE

Animal No 41 Cell tests before immunization was negative At 8 days after immunization, the animal did not show signs of EAE but the cell test was positive After 10 days pareses developed On the 13th day the test was again negative, clinical signs and histology then being positive

In four experiments, the results of blood cell tests were compared with those of thoracic duct lymph cell tests In two of these cases (Nos 51 and 52) both blood and lymph cell tests were negative—neither of these animals, examined 7 and 9 days after immunization resp, presented signs of the disease In the other two cases (Nos 61 and 62) both blood cell and thoracic lymph cell tests were positive These animals were examined 8 and 10 days after immunization, the former showing no signs of EAE, the latter exhibiting both clinical and histological signs A good agreement between white blood cell and lymph cell tests was thus noted

D) *Animals immunized against pig kidney tissue* Tests performed after intervals of 9 12 days were all negative

3 *Rat experiments* As stated previously by us (1962 a), we had great difficulties in obtaining clinical and even histological signs of EAE in

the rat strain used In the present material no cases of EAE were manifest It was possible, however, with thoracic duct lymph cells from injected animals to provoke degeneration phenomena in the glia cultures, comparable to those obtained rabbit cells Table 3 briefly summarizes our results Lymph from normal rats did not produce this effect

TABLE 3
Rat Material

Animal number	Days after immunization	Results of cell test	
		after 24 hrs	after 48 hrs
1	9	0	0
40	10	0	0
41	10	0	0
42	10	0	++
49	10	++	++
50	10	0	0
31	12	+	++
32	13	++	++
43	15	0	0
44	15	++	++
45	15	+	++
51	15	++	++
52	15	+	+
46	20	0	++
60	22	0	0
30	23	++	++

4 *Killing the Lymph Cells*

In a ew experiments, rat or rabbit lymph cells, collected through the thoracic duct and found effective in previous tests, were put into the deep-freeze (—22° C) for a few days This treatment killed the cells

DISCUSSION

... a parallel phenomenon has been described in relation to homograft reaction In the latter experiments, however, the immunologic activity was directed against strain- or species specific antigens—in our experiments against organ-specific antigens With spinal cord tissue from pigs, the immunizations were made in rabbits and tested on rat cells The immunologic state of the rabbits can be described as a brain "allergy" resulting in EAE formation It is not unlikely that the active lymph cells damage glia cells also *in vivo* It is

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Animal No 41 Cell tests before immunization was negative At 8 days after immunization, the animal did not show signs of EAE but the cell test was positive After 10 days pareses developed On the 13th day the test was again negative, clinical signs and histology then being positive

In four experiments, the results of blood cell tests were compared *with those of thoracic duct lymph cell tests In two of these cases* (Nos 51 and 52) both blood and lymph cell tests were negative—neither of these animals, examined 7 and 9 days after immunization resp, presented signs of the disease In the other two cases (Nos 61 and 62) both blood cell and thoracic lymph cell tests were positive These animals were examined 8 and 10 days after immunization the former showing no signs of EAE, the latter exhibiting both clinical and histological signs A good agreement between white blood cell and lymph cell tests was thus noted

D) *Animals immunized against pig kidney tissue* Tests performed after intervals of 9–12 days were all negative

3 *Rat experiments* As stated previously by us (1962 a), we had great difficulties in obtaining clinical and even histological signs of EAE in

weeks after a single immunization The possibility cannot therefore be excluded that this animal would develop FAE on a later occasion

About half of the tests performed on immunized rats were positive Sensitized lymph cells were thus present also in these animals nevertheless no FAE developed

It is perhaps possible to fit the data obtained into various hypotheses We feel that the most likely explanation is the following About 8–10 days after immunization of the animal, sensitized lymph cells appear in the circulation After a few more days clinical signs of the disease develop Circulating sensitized cells then disappear from the blood stream into the brain and spinal cord tissue and a negative test results

SUMMARY

Living white blood cells or thoracic duct lymphocytes taken from rabbits or rats immunized with pig spinal cord attack neonatal rat glia cells kept in tissue culture As a result the glia cells are destroyed This cytopathic effect is found a few days before the onset of the EAE disease When EAE develops the cytotoxicity of the cells disappears

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generally agreed that the antigen responsible for EAE immunization is made up of myelin or fraction of myelin Hence, to obtain an effect on glia cells seems to represent the exceptional finding It should be remembered, however, that the neo-natal glia cells take an active part in myelin-forming processes, and may reasonably be thought to contain myelin antigens *Olmsted* (1961) has recently shown that not only oligodendroglia cells but also astrocytes take part in myelinization in cultures from neo-natal rat brains We have not yet tried to study the toxic effect of sensitized lymph cells on adult glia tissue

It should be noted that the lymph cells are able to attack glia cells without the presence of complement This is in agreement with the findings of, among others, *Goverts* (1960), *Rosenau & Moon* (1961) and *Klein & Sjogren* (1960) The lymph cells must be alive to be effective in the experimental set-up we have used The lymph cell agglutination, observed by us and by *Koprowski & Fernandes* (1962), may play an important rôle in the cytopathic effect

Immunized rabbits may show a negative test In the present material this occurred under the following conditions 1) Before the end of the first week, all tests performed so far were negative This was to be expected as a state of hypersensitivity does not arise until at this stage 2) One animal, which never developed EAE, was negative in the cell test This is also to be expected 3) Some animals—approximately half —which developed EAE were also negative This is at first astonishing The observations made on animals No 41 and 43 may help to explain it, as it was apparent in those animals that a positive test may turn negative a few days later Animal No 41 showed a positive test just before development of EAE, but already 3 days after the onset of clinical signs of EAE, the test was again negative A likely hypothesis is that immunologically active cells are withdrawn from the circulation at the development of the disease, possibly by an absorption into the nervous tissue This idea would agree with the theory suggested by *Waksman* (1959) Animal No 40 showed no clinical signs of EAE 8 days after immunization, but the histological picture showed an unusually strong cellular infiltration in the brain This animal had a negative test

Positive tests were found not only in animals with a manifest EAE In 6 tests, positive results were obtained without signs of EAE 5 of these were made during days 8 10, that is a few days before the expected onset of the disease This finding agrees with *Stone's* (1961) observation that at EAE transfer experiments between guinea pigs, the best results were obtained a few days before the onset of the disease *Astrom & Waksman* (1962) obtained successful transfer in rabbits with lymph node cells already before the appearance of clinical signs of EAE In the sixth case, a positive test was found on the 21st day after immunization but no signs of EAE were present Our experience, however, is that in a few instances EAE may develop extremely late—up to 6

INFECTION AND MALIGNANT TUMOURS

A Comparison between the Effect of Phage Lysates of Haemolytic Streptococci and "Coley Mixed Toxins" on Brown-Pearce Carcinoma

By

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In the latter half of the 19th century several communications appeared on the regression of malignant tumours in connection with acute infections, usually erysipelas (3, 2, 6).

In 1892 *W. B. Coley* commenced treatment of patients suffering from malignant diseases with a non-infectious preparation of haemolytic streptococci and Bacillus prodigiosus (Serratia marcescens). The reason for including B. prodigiosus was the observation that the virulence of the streptococci seemed to increase if they were associated with B. prodigiosus in their proliferating stage (18). Preparations of killed haemolytic streptococci alone were only used for a short period, as they appeared to be without any effect. "Coley Mixed Toxins", however, have not acquired importance in the treatment of human tumours, as no tumour-inhibiting effect has ever been convincingly demonstrated in human cancer. A comprehensive survey of the treatment with "Coley Mixed Toxins" was published by *Nauts et al.* in 1953.

Investigating the Shwartzman reaction, *Gratia & Linz* in 1931 showed that a transplantable guinea pig sarcoma often became necrotic after injection of a filtrate of a culture of Escherichia coli, without any sensitizing injection having been given.

During the next few years, this demonstration led to numerous experiments on transplantable tumours in mice, using toxins from various Gram-negative bacteria (22).

Shear et al. isolated polysaccharides from E. coli and S. marcescens in 1936 and 1943, respectively. The polysaccharides had the same effect on mouse tumours as the unpurified preparations from Gram-negative bacteria (20, 22). The S. marcescens polysaccharide was tested on malignant tumours in human subjects, but no effect could be obtained corresponding to the haemorrhagic necrosis which could be produced in experimental mouse tumours (1, 11, 17, 19).

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mary carcinomas and methylcholanthrene induced tumours in mice and on Rous sarcoma in chickens

In the present investigation, "Coley Mixed Toxins" from the New York Cancer Research Institute were compared with phage lysates of haemolytic streptococci. The comparison was carried out by means of Brown Pearce carcinoma in young rabbits

MATERIAL AND METHODS

Young male rabbits of a white Danish breed ("Copenhagen Whites" from "Hvide sten" the breeding farm of Statens Seruminstitut) were used in the experiments. All animals were between 7 and 9 days old at time of transplantation

Transplantation to the rabbits was by intraperitoneal injection of a cell sus[illegible]

[illegible] following were used in the treatment

1) "Coley Mixed Toxins" made available by the New York Cancer Research Institute. The toxins were prepared from Serratia marcescens and Streptococcus pyogenes and the inhibitory activity towards mouse tumours and the sterility were tested by the New York Cancer Research Institute

2) Lysates of haemolytic streptococci group A type 12 no 3465 (local isolation number). The bacterial culture was lysed by "sewage" phage and filtered sterile through a Berkefeld filter (5). The lysates were prepared by Dr *Ebbe Kjems* Statens Seruminstitut

3) Human serum broth was used as placebo (13)

All injections were given intraperitoneally. The first injection was on the 2nd day after transplantation; the subsequent injections were given either with constant dosage three times a week for three weeks or with increasing dosage every day for a total of 20 days

The general condition of the animals was judged on their increase in weight and on their [illegible] days

The [illegible] as ea[illegible] exper[illegible] the 25th and 35th day after transplantation

Autopsy of all the rabbits was carried out with the same technique and by the same examiner. On the basis of the autopsy findings the animals were divided into three groups according to the occurrence of metastases (A) and into three groups according to the quantity of tumour tissue (B) (5)

A 1 Tumour growth restricted to peritoneum
2 Tumour growth in peritoneum and invasion of diaphragm to pleura
3 Metastases at one or more sites

B 1 No or little tumour tissue
2 Moderate quantity of tumour tissue
3 Large quantity of tumour tissue

RESULTS

In the *first series of experiments*, the animals were treated according to the scheme which in previous experiments with bacterial lysates had given the best inhibition of the Brown Pearce carcinoma (4). The largest dose which did not affect the general condition of the animals was used for the treatment

A total of 25 young rabbits were treated with Coley's toxins at con-

Zahl et al in 1945 studied 32 different strains of haemolytic strepto cocci, 25 belonging to group A, 2 to group B, and 5 to group C, using a technique developed on the basis of the effect of Gram-negative bacteria on mouse tumours They failed to demonstrate any tumour in hibiting effect of the streptococci

In 1955, *Koshimura et al* reported that the take of Ehrlich ascites carcinoma could be prevented, if the tumour cells were incubated together with living haemolytic streptococci before transplantation The inhibiting effect could not be demonstrated with heat-killed haemolytic streptococci, nor with living staphylococci, pneumococci or E coli The same group of investigators demonstrated in 1960 that a cell-free extract of streptococci could also prevent the take of Ehrlich ascites carcinoma

In 1958, *Jordan et al* demonstrated that a transplantable mouse leukaemia could be inhibited by infection with haemolytic streptococci, if the infection occurred less than 72 hours after transplantation

In 1959, *Ginsburg* showed that various proteolytic enzymes which by themselves did not damage the cells of Ehrlich ascites carcinoma, could lyse such cells if the latter had previously been exposed to various streptococcal haemolysins The effect of streptolysin O was blocked by ascites fluid or normal rabbit serum, whereas these had no effect on streptolysins S and D

Christensen & Kjems showed in 1959 that a bacteria-free lysate of haemolytic streptococci, produced by lysing the bacteria with streptococcal phage, could inhibit growth of and metastases from Brown Pearce carcinoma in young rabbits

The above-mentioned experiments with toxins from Gram negative bacteria, together with the old clinical observation of the antagonism between erysipelas and cancer, led *Havas et al* to investigate the tumour-inhibiting effect of a total of 126 preparations of S marcescens, haemolytic streptococci and mixed preparations of the "Coley Mixed Toxins" type The results were compared *with the results of experi*mental infections with the same bacteria The experimental technique was based on the experiments mentioned above with mouse tumours which were inhibited by toxins from Gram-negative bacteria The results were published in 1958 and provided a valuable confirmation of the earlier findings Both purified and unpurified preparations of S marcescens inhibited Sarcoma 37 in ICR Swiss mice A mixed preparation of the type "Coley Mixed Toxins" showed the most pronounced effect in proportion to toxicity Preparations of haemolytic streptococci alone, as well as infections produced *by* haemolytic streptococci, had no appreciable effect on tumour growth

In 1961, *Havas et al* reported that mixed preparations could inhibit Sarcoma 37 and Krebs-2 carcinoma *in mice*, while *no effect* was *found* on three other transplantable mouse tumours, on spontaneous mam-

control animals, either in the presence of metastases or in the quantity of tumour tissue (Fig 1 A and 1 B)

A total of 28 young rabbits received treatment with bacterial lysate in constant dosage In most cases, *with the preparations used, the largest dose* not affecting the general condition of the animals was 5 ml, but 2 5 ml and 10 ml were also used in some cases Two of these 28 animals died before the 12th day after transplantation and were therefore excluded from the material A considerable proportion of the remaining animals showed pronounced inhibition of tumour growth (Fig 1 A and 1 B)

In the *second series of experiments* the animals received increasing doses, this being the system of dosage used by *W B Coley* and now recommended by the New York Cancer Research Institute (16)

The rapid growth and dissemination of the Brown Pearce carcinoma in the young rabbits necessitated a rapid increase in dosage

Treatment with "Coley Mixed Toxins" was instituted with 0 05 ml, the dose increasing to 1 0 ml in the course of 20 days Treatment with bacterial lysate was introduced with 0 75 ml and terminated with 15 ml

A total of 18 animals were treated with "Coley Mixed Toxins" in increasing doses All of them survived long enough to provide comparison with the control animals The treated animals did not differ from the control animals as to presence of metastases and quantity of tumour tissue (Fig 1 A and 1 B)

TABLE 1

	No of rabbits at commencement of experiment	No of rabbits dead before 12th day after transplantation	No of rabbits with adequate observation period	No of rabbits without vital tumour tissue at autopsy
Coley Mixed Toxins constant dose	25	5	20	0
Untreated	45	0	45	0
Bacterial lysate constant dose	28	2	26	8
Placebo treated	28	1	27	0
Total	126	8	118	8

TABLE 2

	No of rabbits at commencement of experiment	No of rabbits dead before 12th day after transplantation	No of rabbits with adequate observation period	No of rabbits without vital *tumour* tissue at autopsy
Coley Mixed Toxins increasing dose	18	0	18	0
Untreated	22	2	20	1
Bacterial lysate increasing dose	18	1	17	0
Placebo treated	18	1	17	0
Total	76	4	72	1

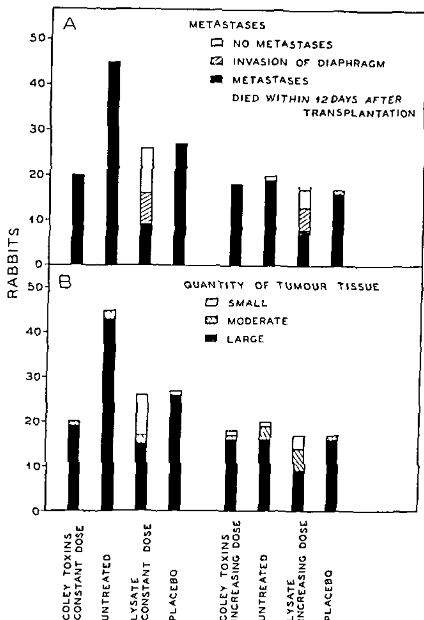


Fig 1 A and B
Metastases and quantity of tumour tissue at autopsy

stant dosage Twelve of these animals received 0 25 ml and 13 received 0 50 ml per injection

Five of the 25 animals died before the 12th day after transplantation *i e* before the tumour had developed sufficiently Four of these five animals had received 0 5 ml and the fifth 0 25 ml per injection 0 5 ml three times a week thus turned out to be too much, although preliminary experiments on young rabbits without tumours had shown that the animals tolerated this dose

The remaining 20 animals did not differ from the corresponding

ditions in which tumour inhibition by lysates of streptococci is clearly demonstrated

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A total of 18 animals received treatment with bacterial lysate in increasing doses One of the animals died before the 12th day after transplantation and was therefore excluded from the material There was inhibition of the tumour growth in a number of the remaining animals Nine of the 17 animals showed no metastases, but in five of these 9 animals the diaphragm had been invaded by tumour tissue, *i e* the animals *showed incipient dissemination of the tumour (Fig 1 A and 1 B)*

The result of treatment with increasing doses of bacterial lysate was poorer than that obtained in the first series using a constant dosage, even though the total dosage was slightly larger in the second series The difference is clearly demonstrated on comparing the findings of vital tumour tissue at autopsy After treatment with constant dosage, 8 of the 26 lysate-treated animals were free from vital tumour tissue After treatment with increasing dosage, all the 17 lysate-treated animals showed vital tumour tissue at autopsy (Table 1 and 2)

DISCUSSION

In the experiments reported here, "Coley Mixed Toxins" had no effect on Brown-Pearce carcinoma under conditions where lysates of haemolytic streptococci had an inhibitory effect on the tumour

Using a technique suitable for demonstrating inhibition of mouse tumour by toxins from Gram-negative bacteria, other workers have been unable to demonstrate tumour-inhibiting effects of haemolytic streptococci, which constitute the one component of "Coley Mixed Toxins" (10, 24)

No inhibition of Brown-Pearce carcinoma could be found with Shear's polysaccharide, produced from Serratia marcescens, the other compo*nent of "Coley Mixed Toxins" (23) On the other hand,* Shear's polysaccharide, like "Coley Mixed Toxins", is effective against a number of mouse tumours (20, 21, 22, 1, 10, 9)

If the results obtained in the above mentioned experiments are compared, the conclusion must be that the effect of *"Coley Mixed Toxins"* in animal experiments is due to toxins from Serratia marcescens The experiments do not exclude the possibility that "Coley Mixed Toxins' contain active substances from haemolytic streptococci, but such substances have shown no appreciable effect under the experimental conditions so far employed, excepting a possible slight increase in the inhibition of Sarcoma 37 (10)

SUMMARY

A comparison is made between the effect of "Coley Mixed Toxins' and the effect of phage-lysed haemolytic streptococci, in young rabbits with Brown-Pearce carcinoma "Coley Mixed Toxins" cannot be shown to have any inhibiting effect on the tumour, under experimental con-

ROM THE INSTITUTE OF CLINICAL BACTERIOLOGY (UNIVERSITY OF LUND) GENERAL HOSPITAL MALMÖ (HEAD PROFESSOR STEN WINBLAD, M D)

A NEW FERMENTATION MEDIUM FOR N. GONORRHOEAE, HAP-MEDIUM. INFLUENCE OF DIFFERENT CONSTITUENTS ON GROWTH AND INDICATOR COLOUR

By

INGMAR JUHLIN

Received 14 vii 62

A bacteriological diagnosis of N gonorrhoeae cannot be based solely on a demonstration of Gram negative diplococci presenting a characteristic colonial appearance and giving a positive peroxidase reaction while incapable of growth on ordinary agar at 22° C A decisive diagnostic creterion must be whether the bacterium is able to ferment dextrose but not maltose, laevulose, or sucrose In view of the medicosocial consequences of a positive diagnosis the evidence on which such a diagnosis is based must be absolutely certain and conclusive

The special growth stimulating factors required by N gonorrhoeae (1, 6), however, have complicated the elaboration of a fermentation medium providing growth in combination with a distinct colour change Several media have been composed which may be used for most N gonorrhoeae strains (1, 3, 10 16 18) but ... or poor grow... of dextrose h... In recent yea... considerably ... owing to the treatment of gonorrhoea with penicillin and other antibiotics (15, 16, 20), in 1958 *Reyn et al* (14) could report a markedly increased frequency, amounting to 40 per cent nonfermenting strains

Many fermentation media are of a semi-fluid consistence (3, 11, 18), and while contained in test-tubes require heavy inoculation of gonococci It is possible only with difficulty to decide whether the inoculate is contaminated or not Nor can the degree of growth be conclusively estimated and related to the degree of dextrose fermentation Up to now, also solid fermentation media have required heavy inoculation for distinct indicator colours to appear (1, 10, 16) and naturally to establish the purity of a culture under such conditions must involve difficulties

When it was found that certain strains were incapable of fermenting

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MATERIALS AND METHODS

Test Strains

The test strains used for preliminary investigation included different strains of N gonorrhoeae N meningitides, and N catarrhalis isolated from routine diagnostic material The final medium was tested with selected atypical strains of N gonorrhoeae and in addition to these with 253 consecutively isolated strains

Inoculation and Incubation

Broth suspensions of pure bacterial cultures were employed as inoculates A platinum loop was charged with the suspension and streaked across a quarter of each medium The plates were incubated at 37° C for 18 22 hours in covered, circular plastic jars in the presence of 8-10 per cent CO_2 As investigations proceeded the importance of sufficiently moist air became increasingly evident, and a water-soaked foam plastic pad was added to each jar (a jar of two litres was calculated to require a pad of 2 × 5 × 5 cm)

Media

All the time while the experiments were being carried out each new medium composition was compared to both "chocolate ascitic agar" and "chocolate ascitic fermentation medium" In addition to these, the fermentation medium in use at the Statens Seruminstitut, Copenhagen referred to below as "fermentation medium CPH", was sometimes employed

Chocolate Ascitic Agar

Broth agar base 1 4 per cent Japan shred agar in human placenta infusion (500 g human placenta and 100 ml aq dest see HAP medium) containing 1 per cent peptone Orthana special 0 2 per cent $Na_2HPO_4 \times 2\ H_2O$ and 0 2 ...

... most of the plasma has been replaced with placenta broth are added and the mixture is kept at 80° C for 20 minutes and frequently shaken It is cooled down to 55° C and mixed with 150 ml of sterile ascitic fluid preheated to 52° C The medium is immediately poured into plastic Petri dishes to form a 4 5 mm layer

Chocolate Ascitic Fermentation Medium"

Prepare 175 ...

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Fermentation Medium CPH

... , 1 per

Evaluation of Medium Compositions

Each ...

dextrose on ordinary media although growth was good and inoculation heavy investigations were initiated in 1957 to obtain better media if possible

A good fermentation medium should.

1 yield good growth of all normal N gonorrhoeae strains even with a moderate or sparing inoculation
2 give a distinct, easily seen but sharply defined colour change in the medium
3 provide sufficient growth to ensure a distinct colour change with, preferably, all atypical strains
4 make it possible to observe contaminating bacterial colonies
5 be easily prepared without different qualities in different batches

Theoretical Motivations for the Choice of Test Substances

The choice of the tested ingredients is partly based on instructions obtained from manuals as regards the various substances generally used and the approximate effect to be expected from these

The importance of choosing a special basic broth was studied, at first partly for economical reasons, placenta infusion broth being compared with other broths

Since certain amino acids have been found to inhibit the growth of N gonorrhoeae (5, 18), a number of peptones of different qualities and partially known structures were chosen

Since agar also may contain inhibitory substances (7), two types were utilized first, ordinary Japanese shred agar, the quality of which may vary considerably from time to time, and next, Bactoagar, pure and standardized

The medium should be of a fairly solid consistence so that it may be easily inoculated on the surface but at the same time it is exceedingly important that the medium readily provides sufficient moisture in order to stimulate bacterial growth The addition of alginate seemed a conceivable answer to this problem

The addition of heated blood results in an opacity of the medium and since it is of a strong brown colour in itself the observations of any other colour will be impaired The problem was therefore to find a transparent solution which did not affect the colour of the medium itself but could be used as a substitute for hematinized blood

Buffering a fermentation medium is not usually recommended but if the intensity of bacterial growth is great enough the medium must be buffered in such a way that the colour indications while being perfectly distinct are limited to a comparatively small area near the pH changing bacterial growth

TABLE 1

Combinations of Various Ingredients Tested

Ingredients	*Amounts tested*	*Combinations* (HAP ↓)
Trypsinbouillon (Pepe)	ad 100 ml	
GC Medium Base Difco	3.6 g	
Peptone UCLAF	2.0 g	
Neopeptone Difco	2.0 g	
Bacto peptone	2.0 g	
Bactotryptose	2.0 g	
Bacto Beefextract	0.3 0.4 g	
Placenta, human	50.0 g	
Proteose Peptone No. 3 Difco	1.0 1.4 2.0 g	
Disodium hydrogen phosphate x 2H2O	0.2 0.24 0.25 g	
Sodium chloride	0.3 0.36 0.5 g	
Aqua destillata	ad 100 ml	
Phenol red Merck	0.04 0.048 0.075 g	
Dextrose	0.2 1.0 1.4 3.0 g	
Agar agar (shred agar Japan)	1.6 g	
Bacto Agar	1.2 1.3 1.5 1.8 2.0 g	
Haemin (bovine) crystallized Sigma	0.0001 0.001 0.004 0.01 g	
Sodium alginate	0.12 g	
Ascitic fluid	25.0 30.0 36.0 ml	
Serum inactivated	10.0 ml	
Haemin (human) McQuarrie & Benlams)	5.0 10.0 15.0 ml	
Bacto Supplement B	1.0 3.0 5.0 ml	
Bacto Yeast Extract	0.5 g	

The intensity of indicator colour change was judged by two criteria first the degree of contrast when compared to the colour of the medium itself second by the area of colour indication in relation to that of bacterial growth 4 = a distinct yellow colour change of the whole area of growth and a surrounding zone of 2 4 mm 3 = a distinct yellow colour in the whole area of growth, 2 = a distinct yellow colour involving about 2/3 of the whole area of growth, 1 = a yellow colour only in the middle of the incubated area or at the site where the richest growth is found, 0 = no visible colour change

Examination of Different Medium Compositions
The Construction and Testing of Medium Varieties

Different combinations and amounts of ingredients were studied in more than 100 medium varieties For practical reasons a complete examination of every mathematically conceivable combination has not been made

In Table 1 most of the examined varieties are represented schematically Among the combinations examined the one described below proved to be the best as regards growth intensity of both normal and atypical strains of N gonorrhoeae, and also as regards distinctly different colour changes, the sizes of single colonies, and the degree of solidity as related to the moisture of the medium

HAP Medium

Placenta infusion fresh human placentas are ground after removal of membranes and umbilical cords The amount thus obtained is weighed and mixed with twice as much aqua dest and kept at 4° C during the night On the next day the mixture is boiled for 20 minutes and paper-filtered yielding a clear fluid which is dispensed into Erlenmeyer flasks and autoclaved at 120° C for 20 minutes

Broth agar base

Placenta infusion	200 ml
Sodium chloride 3 per cent solution in placenta infusion	60 ml
Disodiumhydrogen phosphate x 2 H_2O 2 per cent solution in placenta infusion	60 ml
Proteose peptone No 3 Difco 10 per cent solution in placenta infusion	60 ml
Sodium alginate (acc to BPC 1959)	0 6 g
Bacto agar (Difco)	6 0 g

These ingredients are mixed in each of four flasks and are then autoclaved at 120° C for 20 minutes The broth agar base is cooled to 55° C

HAP-Medium

Broth agar base	380 ml
Ascitic fluid preheated to 52° C	180 ml
Phenol red (Merck) 0 4 per cent water solution	6 ml
Hemin (Sigma) 0 2 per cent water solution	10 ml
Sugar 20 per cent solution in placenta infusion	30 ml

The sterile ingredients are mixed at 52° 55° C and pH is adjusted to 7 7 with 5 N sodium hydroxide The medium is poured into plastic Petri dishes to make a 4 mm layer

Ascitic fluid each fresh batch is sterilized by filtration (Seitz filter EKS2) and compared to earlier batches as regards growthstimulating effect on strains of N gonorrhoeae Streptococcus pyogenes Haemophilus influenzae and Diplococcus pneumoniae

Phenol red Merck 0 4 per cent solution in aqua dest is autoclaved at 120° C for 20 minutes

Hemin bovine, 2 x crystallized. Sigma (in Sweden represented by Chemilia) 0.2 per cent solution in aqua dest 0.15 g hemin crystals are dispersed in 75 ml of aqua dest after which 1.5 ml 3 N sodium hydroxide are added Shake well to dissolve crystals in order to obtain a perfectly clear dark green solution This solution is then sterilized by filtration (Seitz filter EkS2)

Sugar 20 per cent solution of dextrose maltose sucrose or laevulose in placenta infusion The solutions are sterilized by filtration (Seitz filter EkS2)

Discussion of the Ingredients of the Medium

Some observations of the importance of the various constituents deserve to be mentioned

The ability of the medium to stimulate growth is markedly influenced by different infusion materials Horse meat infusion (not included in Table 1) as well as beef extract broth or tryptic digest of horse meat (12) were much inferior to human placenta infusion as regards number and size of colonies At the Statens Seruminstitut, Copenhagen (15), it was found, more or less at the same time, that ox heart infusion while considerably better than ox meat infusion compared unfavourably with human placenta infusion The atypical strains especially grow much better if the infusion is based on human placenta

Comparisons of the various peptones proved Proteose peptone No 3 to be entirely superior to the rest (Neopeptone, Bactotryptose, Bacto peptone, and Peptone UCLAF) According to an analytical scheme in the Difco manual, Proteose peptone contains, among other things, relatively more primary and secondary proteose N, diamino N, and cystine than the others but less ammonia N, peptone N, and free amino N A possible explanation of the superiority of Proteose peptone may consequently be its low content of free amino N or the proportions of its amino acids

Ascitic fluid possesses the best growth-stimulating power and cannot be replaced by Bacto supplement B, Bacto-yeast extract, or a combination of these two Attempts to replace ascitic fluid by other substances are being carried out but have not yet been terminated (Horse serum can not be substituted for ascitic fluid in fermentation media)

There need be no doubt, however, that, next to ascitic fluid, hemin is the most active growth stimulating factor The first hemin solution to be utilized was prepared according to a modification of the method described by *McQuarrie & Beniams* (8), and this hemin solution was standardized colorimetrically The fact that this procedure was both lengthy and laborious, however, led to the adoption of crystallized hemin which meant a great simplification The importance of the thermo stable X-factor in heated blood has been known for a long time but, apparently, crystallized hemin has not previously been employed for this kind of medium Neither Bacto-supplement B nor Bacto-yeast extract, whether alone or in combination, are able to reproduce the effects of this hemin solution

The type and concentration of indicator may effect growth Litmus, bromthymol blue, and neutral red proved unsuitable for several rea-

sons, whereas even a very low concentration of *phenol-red sufficiently* coloured the medium and gave a clearly visible reaction when the sugar was fermented. Attempts to obtain still sharper contrasts by increasing the concentration of phenol-red were unsuccessful, however, owing to depression of gonococcal growth.

The profuse growth developing on the medium necessitated the addition of some buffering substance to prevent a too wide-spread colour reaction when a sugar is being fermented. The plastic Petri dishes applied had diameters of 9 cm and the amount of buffer was adjusted in such a manner as to make room for 4–6 triangleshaped streaks of gonococci. In spite of a distinct yellow colour change under and immediately surrounding the bacterial growth, a zone of unaffected medium should persist between the coloured areas.

EXPERIMENTAL—RESULTS—DISCUSSION

Extensive preliminary experiments provided information about such factors as would contribute to the best fermentation medium. These results led to the elaboration of a new test series for the final tests which were performed on the last 30 or thereabout of the medium varieties.

Ten gonococcal strains showing varying susceptibility to penicillin and streptomycin were selected, suspended in placenta infusion broth, and standardized colorimetrically. These suspensions were then diluted with placenta infusion broth in the following proportions: 1/5, 1/50, 1/250, 1/500, 1/1250 and 1/2500. One loopful from each of the six dilutions of the ten gonococcal strains was used to inoculate one third of each plate. The medium varieties were examined in groups of six to eight and compared with "chocolate ascitic agar" and "chocolate ascitic fermentation medium" (A total of 480–640 inoculations in each of the experimental series).

After incubation at 37° C for 20 hours in the presence of CO_2 the intensity of growth and indication colour with each dilution were estimated and graduated separately fro
calculated for
media were co
of all ten strai
best medium composition of the series in question.

Tables 2 and 3 exemplify the results obtained from one of these test series where the differences between various media depended on the presence or absence of disodiumhydrogen phosphate combined with different kinds and concentrations of hemin.

Table 2 gives the growth and colour change of the 1:5 diluted bacterial suspensions (only 9 strains are represented since one was contaminated). The table is useful in deciding whether any medium composition is wholly unsuitable for a strain or is less able than the rest to produce satisfactory colour in spite of good growth. In Table 3 the points gained by each of the six dilutions with each strain and each medium composition have been added up. The total sum thus obtained indicated *that the* hemin solution prepared in the laboratory is less favourable to growth

TABLE 3

Examples of Evaluation and Registration of Density of Growth and Intensity of Indicator Colour on Medium Varieties

Hemin, McQ & B / Hemin Sigma / $Na_2HPO_4 \times 2H_2O$ (Composition of medium) — N. gonorrhoeae strain	5.0 per cent; 0.2 per cent — colour	growth	5.0 per cent — colour	growth	0.0001 per cent; 0.2 per cent — colour	growth	0.0001 per cent — colour	growth	0.001 per cent; 0.2 per cent — colour	growth	0.001 per cent — colour	growth
R	1	9	2	9	3	9	4	9	5	12	6	12
S	2	5	0	2	1	3	0	1	0	2	0	1
T	6	13	8	13	8	14	7	13	5	12	6	11
U	1	7	1	7	4	9	5	11	5	13	6	14
V	4	6	4	7	3	6	2	6	2	5	2	4
W	0	2	0	3	0	3	1	2	1	4	2	4
X	2	4	0	2	0	3	0	2	0	4	0	1
Y	3	4	2	3	3	4	1	4	3	7	2	4
Z	0	3	0	3	1	4	2	6	2	6	3	8
Total sum	19	53	17	49	23	55	22	54	23	65	27	59
Sum of colour and growth	72		66		78		76		88		86	

Inocula were taken from a dilution series (1/5, 1/50, 1/500, 1/1250, 1/2500) of a dense suspension of N. gonorrhoeae in placenta-infusion broth. One loopful was spread over 1/6 of each plate. Growth and colour were classified from 0–4. Figures indicate the sum of growth or colour "points" from the six dilutions.

TABLE 2

Examples of Evaluation and Registration of Density of Growth and Intensity of Indicator Colour on Medium Varieties

Hemin McQ & B, Hemin, Sigma	Composition of medium: 5.0 per cent			5.0 per cent			0.0001 per cent			0.0001 per cent			0.001 per cent			0.001 per cent		
$Na_2HPO_4 \times 2 H_2O$	0.2 per cent						0.2 per cent						0.2 per cent					
Growth and colour from dilution 1/5 classified as	colour	growth	sum	colour	growth	sum	colour	growth	sum	colour	growth	sum	colour	growth	sum	colour	growth	sum
4	1	1	2	1	2	3	1	1	2	2	2	4	3	4	7	3	4	7
3	2	6	8	1	2	3	3	4	7	1	4	5	1	4	5	1	3	4
2	2	2	4	2	5	7	1	4	5	2	2	4	2	1	3	3	0	3
1	1	0	1	1	0	1	2	0	2	2	1	3	1	0	1	0	2	2
0	2	0	2	4	0	4	2	0	2	2	0	2	2	0	2	2	0	2
Total sum	15*	26	41	12	24	36	17	24	41	17	25	42	20	30	50	21	27	48

* The sum is calculated as follows $4 \times 1 + 3 \times 2 + 2 \times 2 + 1 \times 1 = 15$

Only the inocula from dilution 1/5 in the earlier mentioned dilution series are accounted for Figures indicate the number of strains in the different classification groups The 'Total sum' is the sum of growth or colour "points" for each medium from this dilution

the growth on "chocolate ascitic agar", probably the best medium prepared so far for the cultivation of gonococci, and the growth on the 5 Y variety whereas a corresponding comparison between the number of colonies of 'fermentation medium CPH' and that of the 5 Y-variety presents a striking contrast No less than 5 of the 8 gonococcal strains are virtually incapable of growth on "fermentation medium CPH' under these seeding conditions

As a result of these comparative investigations the composition of the best medium variety could be conclusively established As has been mentioned above it was named HAP medium (Figs 1 and 2)

Later, continuous comparisons were carried out between HAP medium, "chocolate ascitic fermentation medium", and "fermentation medium CPH using 253 consecutively isolated gonococcal strains

The samples are streaked directly onto "chocolate ascitic agar' and incubated at 37° C f ... h
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...

... used colour contrasts were never pronounced enough to permit more than 2 points in the case of this medium

The results from these investigations of 253 consecutively isolated strains of N gonorrhoeae have been summarized in Table 5 The density of growth on HAP-medium and on "chocolate ascitic fermentation medium" are equally good, with approximately 80 per cent of the strains

4

I HAP MEDIUM Chocolate Ascitic Agar and Fermentation Medium CPH

Neisseria gonorrhoeae strain									
M		N		O		P		Q	
0	2500	50	2500	50	2500	50	2500	50	2500
confl	180		270	30	11	confl	60	2	1
confl	180	confl	200	contaminated		confl	60	contaminated	
confl	150	confl	260	*confl*	>400	confl	70	30	7
confl	160	confl	260	confl	confl	confl	80	100	120
confl	>200	confl	290	confl	confl	confl	60	confl	>400
10	0	confl	90	0	0	contam	0	0	4
confl	>200	confl	340	confl	confl	confl	100	*confl*	confl

... th diluted 1 50 and 1/2500 200 colonies at most were counted for strains J M and raised or lowered respectively to the nearest *ten (e g 163 is given as 160)*

than the one based on the crystallized "Sigma" hemin. The stronger of two hemin concentrations yielded the best result. Phosphate is beneficial to growth to some extent but at the same time it exercises a certain inhibitory effect on the colour change in such media as are characterized by the most luxuriant growth.

Taken separately the differences may appear small indeed, owing to the close resemblance in composition between these medium varieties and HAP-medium, but the results indicate the principles on which to base the ideal medium.

The last medium compositions were also studied by counting the number of colonies from a standardized inoculation.

Pure cultures of 8 selected gonococcal strains were used to charge a little less than half a loopful with bacteria which were then suspended in 5 ml of placenta infusion broth. Two dilutions were made of this broth: 1/50 and 1/2500. By means of a 0.25 ml pipette 0.2 ml of each dilution were transferred to Petri dishes containing different medium varieties and the inoculate was then distributed over the whole area using a bent glass rod. After incubation for 20 hours at 37° C in the presence of CO the colonies were counted. Four of the strains (J–M) numbered 200 colonies at the most, the other four (N–Q) 400 colonies at the most (figures have been rounded off to nearest ten, see table).

The results have been summarized in Table 4. The addition of alginate would not seem to affect the growth in this investigation. An increased amount of hemin means a certain increase in the number of colonies, too. In most cases this action is insignificant but with one of the strains (strain Q), a threefold increase of the amount of hemin has a notably stimulating effect on both dilution 1/50 (from 30 colonies to almost confluent growth) and dilution 1/2500 (from 7 to more than 400 colonies). The advantages of the new HAP-medium is best seen in connection with gonococcal strains of this very type.

Only small differences can be detected from a comparison between

Density of Growth of Eight Selected Strains of N. gonorrhoeae on ...

Variants of HAP medium				Neisseria gonorrhoeae strain					
	Na_2HPO_4 per cent	Alginate per cent	Haemin per cent	J 50	J 2500	K 50	K 2500	L 50	
1			0.001	confl	confl	confl	>200		
2		0.1	0.001	contaminated		confl	contam	confl	co…
3	0.2	0.1	0.001	confl	>200	confl	confl	confl	
4	0.2	0.1	0.002	confl	>200	confl	50	confl	
5	0.2	0.1	0.003	confl	>200	confl	>200	confl	
Ferment. medium CPH				confl	>200	0	10	>200	co…
Chocolate ascitic agar				confl	confl	contam	>200	confl	

Inocula prepared from suspensions of ten colonies in five ml of placenta inf…
400 at most for strains N–Q. For convenience the values obtained have…

TABLE 5

Density of Growth and Intensity of Indicator Colour of 253 Consecutively Isolated Strains of N gonorrhoeae on HAP Medium Fermentation Medium CPH and Chocolate-Ascitic Fermentation Medium

Degree of growth and colour intensity		HAP medium: Dextrose colour	HAP medium: Dextrose growth	HAP medium: Laevul growth	HAP medium: Malt growth	HAP medium: Sacch growth	Ferment medium CPH: Dextrose colour	Ferment medium CPH: Dextrose growth	Ferment medium CPH: Malt growth	Chocolate ascitic ferment medium: Dextrose colour	Chocolate ascitic ferment medium: Dextrose growth	Chocolate ascitic ferment medium: Laevul growth	Chocolate ascitic ferment medium: Malt growth	Chocolate ascitic ferment medium: Sacch growth
4	Number	187	199	201	204	203	11	67	58		199	204	211	209
	Per cent	73.9	78.7	79.4	80.6	80.2	4.3	26.5	22.9		78.7	80.6	83.4	82.6
3	Number	27	34	40	38	34	33	19	25		35	36	32	34
	Per cent	10.6	13.4	15.8	15.0	13.4	13.1	7.5	9.9		13.8	14.2	12.6	13.4
2	Number	24	16	8	5	12	34	21	15	179	9	9	7	9
	Per cent	9.5	6.3	3.2	2.0	4.8	13.4	8.3	5.9	70.7	3.6	3.6	2.8	3.6
1	Number	10	3	1	6	2	27	39	41	50	2	3	2	1
	Per cent	4.0	1.2	0.4	2.4	0.8	10.7	15.4	16.2	19.8	0.8	1.2	0.8	0.4
0	Number	5	1	3	0	2	148	107	114	24	8	1	1	0
	Per cent	2.0	0.4	1.2	0	0.8	58.5	42.3	45.1	9.5	3.2	0.4	0.4	0

The inoculum one loopful of a suspension of ten colonies in 1.0 ml broth, was spread over a quarter of each plate



Fig 1

Three strains of N *gonorrhoeae on HAP medium one normal (top) and two atypical* poorly growing strains (left and right) Note the different size of the colonies (Incubated at 37° C for 48 hours in the presence of CO_2)

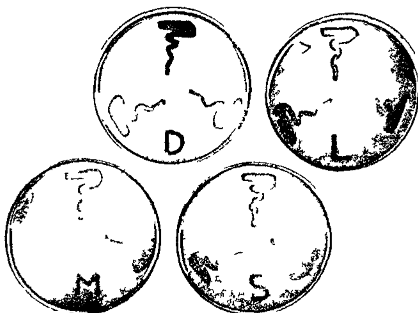


Fig 2

Strains of N gonorrhoeae (right) N sicca (top) and N meningitidis (left) on HAP medium with dextrose (D) laevulose (L) maltose (M) and sucrose (S) (Incubated at 37° C for 18 hours in the presence of CO_2)

TABLE 6

A Detailed Report of such N. gonorrhoeae Strains as Showed none or merely Weak Dextrose Fermentation on Chocolate Ascitic Fermentation Medium or HAP Medium when Primarily Diagnosed, Followed by the Results of a Second Cultivation of some of the Strains

	Results on primary isolation										Results on control									
	Colour classified as					Growth classified as					Colour classified as					Growth classified as				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Chocolate ascitic fermentation medium	24	–			–	7	2	4	3	8	6	1	4	–	–	3	–	–	–	8
HAP medium	2	5	5	1	11	1	–	5	2	16	–	–	3	–	8	–	–	–	–	11
Chocolate ascitic fermentation medium		50	–		–	–	–	5	17	28	1	1	8	–	–	–	–	–	2	8
HAP medium	3	4	10	9	24	–	2	6	14	28	–	1	1	1	7	–	–	–	3	7
HAP medium	5	–	–	–	–	1	1	–	3	–	1	–	1	–	2	–	–	1	1	2
Chocolate ascitic fermentation medium	2	3	–	–	–	1	–	–	3	1	2	–	2	–	–	1	–	1	–	2
HAP medium	–	10	–	–	–	–	2	7	1	–	–	–	1	–	3	–	–	–	2	2
Chocolate-ascitic fermentation medium	5	4	1	–	–	2	–	5	2	1	1	–	3	–	–	1	–	–	1	2

growth has been considered maximal and with an additional 13 per cent nearly maximal On "fermentation medium CPH", however, only 25 per cent attained maximal growth, and a further 8 per cent nearly maximal growth, while no less than 42 per cent of the gonococcal strains are unable of any growth whatsoever with ordinary inoculation

The most important distinctions between the different media though are found when we compare the depth of their colour indication The colour given 2 points on HAP-medium is much more easily discernible than the correspondent 2-point colour on "chocolate ascitic fermentation medium" *owing to the transparency and better contrast effects of* HAP medium To obtain a proper comparison it is thus necessary to take points 2, 3, and 4 on HAP-medium together It then appears that 95 per cent of the strains show a very clear colour change on HAP medium as compared with 70 per cent on "chocolate ascitic fermentation medium", and merely 30 per cent on "fermentation medium CPH" The colour changes given 1 point only constitute 4 per cent on HAP medium, and approximately 20 per cent on "chocolate ascitic fermentation medium", but here, too, it must be borne in mind that the 1-point colour is much better seen on HAP medium than on "chocolate ascitic fermentation medium"

These gonococcal strains which failed to present a colour change in this routine investigation amount to 2 per cent on HAP medium, 10 per cent on "chocolate ascitic fermentation medium", and 59 per cent on "fermentation medium CPH"

Even for routine diagnostic purposes the new HAP-medium has thus shown its superior qualities in spite of the fact that the density of the seeding suspension has been chosen explicitly so as to give the ideal density of colonies for purposes of resistance testing (by a diffusion method using antibiotic discs of paper (2), on "chocolate ascitic agar) "Ideal density of colonies" in this case implies closely arranged, nearly confluent colonies giving a strong impression of a granular surface

Some of the gonococcal strains of the routine investigation reported above, which *did not grow or gave a 1-point-colour only*, have been submitted to further study to see whether the medium is unsuitable for these strains or whether the unsatisfactory results were purely accidental

Unfortunately, not all of these strains have been accessible to observation Table 6 presents the results of the primary cultivation as well as the results of a secondary investigation of the strains at disposal In 24 of the gonococcal strains which showed no colour change on *"chocolate ascitic fermentation medium", the reason in* seven cases was absence of growth but on HAP-medium all except one grew, and all except two gave a colour change Out of these 24 strains, eleven were inoculated a second time but even then 3 were incapable of growth on "chocolate ascitic fermentation medium and three others gave no colour change although growth was satisfactory But on HAP-medium

gave good growth and indication colour on HAP medium whereas one did not grow on 'chocolate ascitic fermentation medium"

The conclusion must be that out of 253 investigated strains, one strain gave no colour change on HAP-medium after 20 hours of incubation and one strain, inaccessible to subsequent re-examination, did not grow

Since one of the control media was 'fermentation medium CPH' which was in use at the Statens Seruminstitut, Copenhagen, Dr A Reyn who is director of the genococcal department there did kindly subject the new HAP medium to further examination Eight specially selected gonococcal strains, consisting of 2 normal and 6 atypical, poorly growing strains were employed, and two dilutions, 1/1 and 1/10, were prepared of each strain The undiluted suspension 1/1, contained 10^8 10^9 bacteria per ml The plates were incubated as usual, colonies were counted, and their size and intensity of colour estimated

The results are presented in Table 7, showing the numbers of colonies on HAP medium, HAP medium without hemin and "fermentation medium CPH' All of the strains exhibit excellent growth on HAP medium, averaging approximately 500–600 colonies with dilution 1/1 Removal of

TABLE 8

[illegible] of *N gonorrhoeae*

N gonorrhoeae strain	Dilut	HAP medium		HAP medium without hemin		Ferment medium CPH	
		Colony diameter		Colony diameter		Colony diameter	
		smallest mm	largest mm	smallest mm	largest mm	smallest mm	largest mm
A	1/1	1,0	2,0	0,5	1,0		–
	1/10	1,0	2,0	0,5	1,0		0,25
B	1/1	0,5	2,0	0,5	1,5		1,0
	1/10	1,5	2,5	0,5	1,0		0,5
C	1/1	0,25	1,0	0,25	0,5	–	–
	1/10	0,25	1,0		0,5		–
D	1/1	0,5	1,0		1,0		–
	1/10	0,5	1,0	–			–
E	1/1	0,5	1,5		0,5		–
	1/10	0,5	2,0		–	–	–
F	1/1	0,5	2,5	0,5	2,0		–
	1/10	0,5	2,0	0,5	1,0	–	–
G	1/1	0,25	0,25	0,5	0,5	–	–
	1/10	0,25	0,25	0,5	0,5	–	–
H	1/1	0,25	0,25	0,25	0,5		–
	1/10	0,25	0,25	0,25	0,25		
Average value	1/1	0,5	1,3	0,4	0,9		(0,1)
	1/10	0,6	1,4	0,5	0,7	–	(0,1)

5*

all of the strains grew fermenting dextrose with a resulting colour change

All of the 50 strains which gained 1 point for colour on "chocolate ascitic fermentation medium" grew satisfactorily, and on HAP-medium 43 produced a distinct colour change, 4 strains a weak one, and 3 strains no colour change Ten of these strains have been controlled and found to give good growth but, in one case, no indication colour developed on "chocolate ascitic fermentation medium"

As stated above, five strains developed no colour change on HAP-medium on routine diagnosis Out of these, two strains grew poorly or not at all, while "chocolate ascitic fermentation medium" gave no growth in one case and no colour change in another Four of these strains were re-examined with the result that three showed good growth and colour on HAP-medium whereas the fourth merely developed moderate growth and no colour after 20 hours of incubation On "chocolate ascitic fermentation medium" two strains were still incapable of colour change, one simply because it did not grow

Ten strains had gained 1 point for colour on HAP-medium, none of them growing entirely satisfactorily On "chocolate ascitic fermentation medium" 5 of these gave no colour change, in two cases because of absence of growth Four of these strains were re-examined and found to

TABLE 7

Results Obtained from Counting the Number of Colonies on HAP Medium HAP Medium without Hemin and 'Fermentation Medium CPH', as Carried out at Statens Seruminstitut Copenhagen on two 'Normal' Strains (A–B) and six Selected Atypical Strains (C–H) of N gonorrhoeae

N gonorrhoeae strain	Dilut	HAP medium		HAP medium without hemin		Ferment medium CPH	
		Dextrose	Maltose	Dextrose	Maltose	Dextrose	Maltose
A	1/1	283	414	171	385	0	0
	1/10	38	48	26	31	0	1
B	1/1	680	720	107	27	0	3
	1/10	76	79	5	6	2	0
C	1/1	125	145	5	16	0	0
	1/10	9	21	0	1	0	0
D	1/1	458	590	0	1	0	0
	1/10	42	77	0	0	0	0
E	1/1	526	717	1	0	0	0
	1/10	59	72	0	0	0	0
F	1/1	354	459	20	118	0	0
	1/10	38	52	4	3	0	0
G	1/1	1486	1526	1474	1730	0	0
	1/10	147	182	89	190	0	0
H	1/1	452	540	374	590	0	0
	1/10	32	52	24	43	0	0
Average number	1/1	546	639	269	358	0	0.4
	1/10	55	73	19	34	0.3	0.1

give good growth and indication colour on HAP medium whereas one did not grow on "chocolate ascitic fermentation medium'

The conclusion must be that out of 253 investigated strains, one strain gave no colour change on HAP medium after 20 hours of incubation and one strain, inaccessible to subsequent re-examination, did not grow

Since one of the control media was "*fermentation medium CPH*' which was in use at the Statens Seruminstitut, Copenhagen, Dr A Reyn who is director of the genococcal department there did kindly subject the new HAP-medium to further examination Eight specially selected gonococcal strains consisting of 2 normal and 6 atypical, poorly growing strains were employed, *and two dilutions, 1/1 and 1/10*, were prepared of each strain The undiluted suspension, 1/1, contained 10^8–10^9 bacteria per ml The plates were incubated as usual, colonies were counted, and their size and intensity of colour estimated

The results are presented in Table 7, showing the numbers of colonies *on HAP medium HAP medium without hemin and "fermentation medium CPH*' All of the strains exhibit excellent growth on HAP-medium, averaging approximately 500–600 colonies with dilution 1/1 Removal of

TABLF 8

on HAP Medium HAP
s Carried out at Statens
and six Selected Atyp

N gonorrhoeae strain	Dilut	HAP medium		HAP medium without hemin		Ferment medium CPH	
		Colony diameter		Colony diameter		Colony diameter	
		smallest mm	largest mm	smallest mm	largest mm	smallest mm	largest mm
A	1 1	1 0	2 0	0 5	1 0	–	–
	1 10	1 0	2 0	0 5	1 0		0 2>
B	1/1	0 5	2 0	0 5	1 5	–	1 0
	1/10	1 5	2 5	0 5	1 0		0 5
C	1 1	0 2>	1 0	0,25	0 5	–	
	1 10	0 25	1 0	–	0 5	–	–
D	1 1	0 5	1 0		1 0	–	–
	1 10	0 5	1 0			–	–
F	1/1	0 5	1 5		0 5	–	–
	1 10	0 5	2 0	–	–		–
F	1 1	0 5	2 5	0 5	2 0		–
	1 10	0 5	2 0	0 5	1 0	–	–
G	1 1	0 2>	0 2>	0 5	0 5		–
	1 10	0,2>	0 25	0 5	0 5	–	–
H	1 1	0.2>	0 2>	0 75	0 5		–
	1/10	0 25	0 25	0,2>	0,2>	–	–
Average value	1 1	0 5	1,3	0 4	0 9	–	(0 1)
	1 10	0 6	1 4	0 5	0 7	–	(0 1)

TABLE 9

Results Obtained from Counting the Number of Colonies on Variants of HAP Medium as Carried out at Statens Seruminstitut, Copenhagen, on two Normal Strains (A–B) and six Selected Atypical Strains (C–H) of N. gonorrhoeae

		HAP medium		Variants of HAP medium					
Hemin per cent		0.003		0.003					
Alginate » »		0.6						0.6	
N. gonorrhoeae strain	Dilut	Dextrose	Maltose	Dextrose	Maltose	Dextrose	Maltose	Dextrose	Maltose
A	1/1	283	414	451	590	82	138	171	385
	1/10	38	48	49	52	11	18	26	31
B	1/1	680	720	924	760	934	447	107	27
	1/10	76	79	67	84	60	60	5	6
C	1/1	125	145	149	227	78	116	5	16
	1/10	9	21	19	31	1	12	0	1
D	1/1	458	590	514	600	16	45	0	1
	1/10	42	77	64	79	1	2	0	0
E	1/1	526	717	665	1031	14	53	1	0
	1/10	59	72	70	86	2	7	0	0
F	1/1	354	459	600	673	121	457	20	118
	1/10	38	52	96	92	29	11	4	3
G	1/1	1486	1526	1424	1368	1190	1458	1474	1730
	1/10	147	182	161	181	126	212	89	190
H	1/1	452	540	612	510	430	656	374	590
	1/10	32	52	49	48	33	37	24	43
Average number	1/1	546	639	667	720	291	421	269	358
	1/10	*55*	*73*	*72*	*82*	*33*	*49*	*19*	*34*

the hemin reduces the number to about half its former value. "Fermentation medium CPH", on the other hand, merely produced single colonies of the two normal strains.

The measurements of the colonial diameters are reported in Table 8, showing a fairly wide range of 0.25–2.5 mm between different strains, the average being 0.5 mm for the smallest and 1.4 mm for the largest colonies. Here, too, the importance of hemin is illustrated by the fact that only one of the strains developed colonies of a maximum diameter of 2.0 mm in the absence of hemin, the average being only 0.4 mm for the smallest and 0.9 mm for the largest colonies.

The importance of hemin and alginate in different varieties of HAP-medium was also studied—colonies were counted, yielding the results reported in Table 9. Hemin, as had already been established, was shown to be an essential growth-stimulating factor. In contrast alginate proved to have slightly inhibitory effects, especially pronounced in the medium variety lacking in hemin as well, where three strains showed none or very scanty growth. However, the difference between the original HAP-

TABLE 10

Results Obtained from a Determination of the Intensity of Indicator Colour Change on Variants of HAP Medium and Fermentation Medium CPH', as Carried out at Statens Seruminstitut Copenhagen, on two Normal Strains (A–B) and six Selected Atypical Strains (C H) of N Gonorrhoeae

	HAP medium	Variants of HAP medium			Ferment medium CPH
Haemin per cent	0 007	0 0[illegible]3			
Alginate " "	0 6			0 6	
N gonorrhoeae strain					
A	4	4	2	4	0
B	4	4	4	4	0
C	3	4	0	0	0
D	4	4	0	0	0
E	4	4	0	0	0
F	4	4	2	4	0
G	3	3	3	3	0
H	0	0	0	0 5	0
Average value	3 25	3 4	1 4	1 9	0

medium and the HAP-medium without alginate is small and of no practical consequence

As may be seen from Table 10 the intensity of colour change was given 3 or 4 points on HAP-medium in the case of 7 of the 8 strains, but one of the strains gave no definite colour change Unfortunately, however, no information is available whether the strain can ferment dextrose and produce a colour change if inoculation is increased so as to give confluent growth

Dr A Reyn's investigations resulted in a substitution of HAP-medium, for the previously used "fermentation medium CPH' at the gonococcal department of the [illegible]

In addition to this, a [illegible]

mark, Norway, Finland, [illegible]

cultivation and resistance tests have redommended a switch on to HAP-medium as a fermentation medium for gonococci

SUMMARY

When new media *for the cultivation of* gonococci are being worked out, special regard must be paid to atypical strains characterized by scanty growth, small colonies, and poor viability [illegible]

[illegible] *must contain all of the growth-stimu-*

TABLE 9

Results Obtained from Counting the Number of Colonies on Variants of HAP Medium, as Carried out at Statens Seruminstitut, Copenhagen, on two Normal Strains (A–B) and six Selected Atypical Strains (C–H) of N gonorrhoeae

Hemin per cent / Alginate » »		HAP medium 0 003 / 0 6		Variants of HAP medium 0 003 / —		— / —		— / 0 6	
N gonorrhoeae strain	Dilut	Dextrose	Maltose	Dextrose	Maltose	Dextrose	Maltose	Dextrose	Maltose
A	1/1	283	414	451	590	82	138	171	385
	1/10	38	48	49	52	11	48	26	31
B	1/1	680	720	924	760	934	447	107	27
	1/10	76	79	67	84	60	60	5	6
C	1/1	125	145	149	227	78	116	5	16
	1/10	9	21	19	31	1	12	0	1
D	1/1	458	590	514	600	16	45	0	1
	1/10	42	77	64	79	1	2	0	0
E	1/1	526	717	665	1031	14	53	1	0
	1/10	59	72	70	86	2	7	0	0
F	1/1	354	459	600	673	121	457	20	118
	1/10	38	52	96	92	29	11	4	3
G	1/1	1486	1526	1424	1368	1190	1458	1474	1730
	1/10	147	182	161	181	126	212	89	190
H	1/1	452	540	612	510	430	656	374	590
	1/10	32	52	49	48	33	37	24	43
Average number	1/1	546	639	667	720	291	421	269	358
	1/10	55	73	72	82	33	49	19	34

the hemin reduces the number to about half its former value "Fermentation medium CPH", on the other hand, merely produced single colonies of the two normal strains

The measurements of the colonial diameters are reported in Table 8, showing a fairly wide range of 0 25–2 5 mm between different strains, the average being 0 5 mm for the smallest and 1 4 mm for the largest colonies Here, too, the importance of hemin is illustrated by the fact that only one of the strains developed colonies of a maximum diameter of 2 0 mm in the absence of hemin, the average being only 0 4 mm for the smallest and 0 9 mm for the largest colonies

The importance of hemin and alginate in different varieties of HAP-medium was also studied—colonies were counted, yielding the results reported in Table 9 Hemin as had already been established was shown to be an essential growth-stimulating factor In contrast alginate proved to have slightly inhibitory effects, especially pronounced in the medium variety lacking in hemin as well, where three strains showed none or very scanty growth However, the difference between the original HAP-

the time when the broth agar base the ascitic fluid and the other ingredients are mixed giving a final concentration of about 0 001 per cent of cocarboxylase

Thus it has been possible with all the strains of N gonorrhoeae which have so far been studied to get a satisfactory growth and an intense colour change using HAP-medium containing 0 001 per cent of cocarboxylase

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16 *Reyn A & Bent on M W* Possible effects of antibiotic treatment on the sensi tivity and growth requirements of Neisseria gonorrhoeae Bull Wld Hlth Org 24 333 1961
17 *Ringert* [illegible]
18 *Thomp* [illegible]
19 *Weed* [illegible] Mimae—A problem in diagnosis and taxonomy Amer J clin Path 35 447 1961
20 *Welsh M* La résistance bacterienne aux antibiotiques Antibiot et Chemother 2 34 1955

lating factors needed by normal, and particularly by atypical, gonococcal strains

This new HAP-medium has been tested with 253 consecutively isolated strains of N gonorrhoeae as well as with several, specially chosen, atypical strains of N gonorrhoeae At the same time, HAP-medium has been compared to "chocolate ascitic agar", "chocolate ascitic fermentation medium", and "fermentation medium CPH" The growth stimulating power of HAP-medium was found to be virtually equal to that of "chocolate ascitic agar" and far superior to that of "fermentation medium CPH" Fermentation reactions are much more readily demonstrable on HAP-medium than on the other two fermentation media

Thanks to the good growth-stimulating qualities of HAP-medium, the routine procedure was simplified, permitting a finer control of the isolated gonococcal strains This became possible when it was found that both fermentation and resistance tests could be carried out on the same bacterial suspension, consisting of 10 gonococcal colonies in 1 ml of placenta infusion broth A loopful of the suspension was streaked across 1/4–1/6 of the four HAP-medium plates with different sugars and the rest of the suspension was poured onto a plate of "chocolate ascitic agar" for resistance test by a diffusion method using 5 different antibiotic discs of paper (2)

This routine procedure thus guarantees that the bacterial strain whose resistance is reported to the clinician, really is N gonorrhoeae, and no fears need arise that strains of lowered suspectibility against penicillin might not be gonococci (4, 19)

Even though long application of HAP-medium may result in the appearance of single strains exhibiting atypical behaviour here too it will be seen from the varioues test series reported above that this new medium nevertheless has proved its reliability, and its advantages for routine diagnosis if compared to media previously used

ADDENDUM

Investigations were continued on more than 1000 strains of N gonorrhoeae with a view to improve the growth and colour intensity of the very few strains that showed a sparse growth and a weak colour change

During the routine diagnostic work a few such strains were found and some others were received from other laboratories In all 12 strains of N gonorrhoeae have been studied which showed a weak or no colour change after an incubation of 20 hours

All of these strains except three showed a perfect colour change after 48 hours of incubation however

By the addition of 2 drops of a 0 1 per cent buffered solution of cocarboxylase to the surface of the HAP medium in the area of inoculation the growth and c lour [illegible] improved with all of the strains except one

[illegible] a desirable degree of indicator [illegible] r cent of cocarboxylase was used

Cocarboxylase (Bérolase [illegible] el) is dissolved in a buffer consisting of 1 0 g sodium acetate, 0 2 g sodium hydroxide and 100 ml sterile water to make a 0 1 per cent solution Of this solution 6 ml are added to the HAP medium at

These bacteriological properties are phage type drug resistance and production of lipase (*Gillespie & Alder* 1952) Interest in the last reaction was aroused by the results of *Jessen & al* (1959b), who on investigating staphylococcal bacteraemia cases found that those caused by strains not producing the enzyme (*i e* strains that were FY negative) had a higher mortality rate than cases caused by strains producing lipase (FY positive)

METHODS

Registration

The cards have been filed according to the hospital and date of the person s birth One strain is represented by one card

The card indicates where the strain was isolated from (nose pathological process etc) its phage type its drug resistance and its production of lipase

During the whole period the laboratory received a total of about 10 000 strains isolated from patients or staff members However in the present survey only one strain from each person is included The strain chosen is the one first isolated from a pathological process Strains from nose and throat are included if they represent

Bacteriological Investigations

Statens Seruminstitut received the specimens either as swabs directly from the hospitals or as pure cultures from other laboratories

Bacteriophage typing of the staphylococci was performed as described by *Rosendal* (1959) Following suggestions from the international committee on phage typing of staphylococci minor changes were introduced Phages 81 and 83A were included in the basic set of phages The pooled bacteriophage suspensions employed contain the following phages Pool A 31B 42B 47C 57 pool B 73 75A 83B 52B pool C 44 51 69 78

Production of lipase was demonstrated as described by *Jessen et al* (1959 a) using *Sierra*s medium (1957)

Resistance to antibiotics was determined in several laboratories (Statens Seruminstitut the L … rhus and various hospital laborat r … disc method (*Jensen & Kiær* 1947 Dra … (*Lund et al* 1951)

T … esistant to the various antibiotics invc … was reported strains with a zone al … e … mm were classified as sensitive If the zone was less than 25 mm the strains were considered resistant

RESULTS

The material comprises 6 932 strains isolated from 6 932 persons

Table 1 shows the distribution of the material over various counties of Denmark

The figures do *not* give any information about the frequency of infections or about the occurrence of epidemics The number of phage typed strains may only reflect local interest in problems concerning hospital infections Furthermore many strains isolated locally may never have been forwarded to the phage typing laboratory

The number of specimens received increased in May, 1960 At this time Sygehusforeningen i Danmark set up an organisation to check up

FROM STATENS SERUMINSTITUT, COPENHAGEN (DEPT OF DIAGNOSTIC BACTERIOLOGY), THE INSTITUTE OF GENERAL PATHOLOGY, UNIVERSITY OF AARHUS THE INSTITUTE OF HYGIENE, UNIVERSITY OF AARHUS AND THE INSTITUTE OF GENERAL PATHOLOGY, COPENHAGEN

STAPHYLOCOCCUS AUREUS STRAINS ISOLATED IN DANISH HOSPITALS FROM APRIL 1ST TO DECEMBER 31ST 1960

By

KIRSTEN ROSENDAL, AKSEL STENDERUP, PEDER HELMS and KNUD RIEWERTS ERIKSEN

Received 3 vii 6

On April 1st, 1960, Statens Seruminstitut began a registration of al phage-typed *Staphylococcus aureus* (= coagulase-positive staphylo cocci) strains isolated from patients and staff members of hospital all over Denmark

The purpose of this registration was to record the epidemiologic occurrence of staphylococci at each hospital From time to time i proved possible to discover epidemics at their very beginning and to inform the local authorities about the situation

This arrangement succeeded because the phage-typing of staphylo cocci was performed in one laboratory only

The centralization was in certain ways a disadvantage The registra tion does not give a true picture of the staphylococcal hospital infec tions, it was not possible to decide whether the infections from which the staphylococci were isolated originated before or after hospitaliza tion, as the specimens were forwarded to the laboratory without infor mation necessary for making this distinction Furthermore, it was impossible to ascertain whether all infections of epidemiological in terest were bacteriologically investigated

As a consequence, thorough studies of epidemics and of the fre quency of infections could not be carried out on the basis of the present material, which comprises specimens received from April 1st to De cember 31st, 1960 However, a continued annual survey will permit the observation of changes in the situation from year to year The re gistration will continue for at least five years

In another respect the centralization was a great advantage It was considered justifiable to regard the strains of the material as epidemio logically unrelated, as they were isolated from about 150 hospitals scattered all over Denmark Therefore the material is suitable for the evaluation of certain bacteriological properties of the strains investi gated—and this is the chief object of the present report

These bacteriological properties are phage-type, drug resistance and production of lipase (*Gillespie & Alder* 1952) Interest in the last reaction was aroused by the results of *Jessen & al* (1959b), who on investigating staphylococcal bacteraemia cases found that those caused by strains not producing the enzyme (*i e* strains that were EY negative) had a higher mortality rate than cases caused by strains producing lipase (EY-positive)

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Production of lipase was demonstrated as described by *Jessen et al* (1959 a) using *Serra's* medium (1957)

Resistance to antibiotics was determined in several laboratories (Statens Serum institut the Universities of Copenhagen and Århus and various hospital laboratories) Two methods were used 1) the paper disc method (*Jensen & Kiær* 1947 *Brøjstel & Erichsen* 1953) 2) the tablet method (*Lund et al* 1951)

The strains were classified as sensitive or resistant to the various antibiotics investigated If the size of the inhibition zone was reported strains with a zone above 25 mm were classified as sensitive If the zone was less than 25 mm the strains were considered resistant

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As a consequence, thorough studies of epidemics and of the frequency of infections could not be carried out on the basis of the present material, which comprises specimens received from April 1st to December 31st, 1960. However, a continued annual survey will permit the observation of changes in the situation from year to year. The registration will continue for at least five years.

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TABLE 2

Clinical Sources of Strains Investigated

Group	Isolated from	Number of strains	Per cent
A	× unknown source	56	0.8
	• pure culture	33	0.5
	+ { swab	227	3.3
	{ pus	954	13.8
	total	1270	18.8
B	++ region stated	439	6.3
	abscesses	1300	18.8
	pustuli	430	6.3
	hands	472	6.8
	cicatrices	344	5.0
	wounds	385	5.5
	combustion	17	0.2
	mastitis	85	1.2
	eczema and skin diseases	12	0.2
	total	3484	50.2
C	eye	119	1.7
	ear	240	3.4
	nose	290	4.2
	mouth and throat	190	2.7
	vagina	24	0.3
	total	863	12.0
D	sinus	23	0.3
	larynx trachea	129	1.9
	bronchii	43	0.6
	sputum	516	7.4
	urine	258	3.7
	faeces	31	0.4
	total	1000	14.0
E	blood	63	1.0
F	spinal fluid	9	0.1
	viscera	42	0.6
	exudate	51	0.7
	osteomyelitis	16	0.2
	joint	34	0.5
	+++ fistulae	99	1.4
	autopsy	1	0.0
	total	252	4.0
	Total number of strains	6932	100

× (unknown source) No information received

• (pure culture) Pure cultur [illegible]

+ (swab and pus) The only ir [illegible]

++ (region stated) It is stated [illegible] been taken [illegible] excepted [illegible]

+++ (fistulae) Comprises fistulae from viscera and ossa

on hospital infections in Denmark. The increase of specimens may be a result of this new activity. A further increase occurred in September 1960, when it was decided to phage type all *Staphylococcus aureus* strains from hospitals, isolated in the various departments in Statens Seruminstitut. Previously the typing had only been done when requested by the hospitals.

TABLE 1

Distribution of the Material on the Various Counties of Denmark

County		Number of specimens	Population registered July 1st 1960
København	(borough)	2403	725 000
Frederiksberg	( )	214	114 500
København	(county)	229	481 000
Roskilde	( )	47	89 800
Frederiksborg	( )	131	180 300
Holbæk	( )	79	127 200
Sorø	( )	91	129 800
Præstø	( )	94	122 200
Bornholm	( )	29	45 500
Lolland Falster	( )	86	132 300
Svendborg	( )	26	149 200
Odense	( )	248	264 400
Vejle	( )	221	213 800
Skanderborg	( )	240	137 900
Århus	( )	1446	220 200
Randers	( )	316	170 100
Ålborg	( )	280	238 800
Hjørring	( )	233	177 600
Thisted	( )	83	85 100
Viborg	( )	130	162 000
Ringkøbing	( )	92	205 500
Ribe	( )	119	185 000
Haderslev	( )	70	72 400
Åbenrå Sønderborg	( )	16	105 500
Tønder	( )	4	42 500
Færøerne	( )	1	34 500
Grønland	( )	4	31 500
Total		6 932	4581 000

Future registration may reveal whether there is any seasonal fluctuation in the number of staphylococcal cases. An enumeration of the staphylococci isolated at Statens Seruminstitut from May to December 1960 seems to bear out the suggestion that an increase did take place during the autumn of 1960.

The processes and regions from which the staphylococci were isolated are given in Table 2. 50 per cent of the strains originated from various pathological conditions of the skin and the subcutaneous tissues (group B), most of these being from abscesses. At least 5 per cent of the strains were isolated from post-operative infections (cicatrices), and

TABLE 4

Percentage Distribution of Phage Patterns and Groups among Strains from Various Clinical Sources

Source	Group A %	Group B %	Mastitis %	Group C %	Group D %	Blood %	Spinal fluid %	Group E %	Total %
Phage type group									
80/81	20	21	35	8	5	21	0	17	17
80	3	4	2	1	3	3	0	3	4
81	4	3	5	3	2	2	0	2	3
52/52A/80	15	17	21	10	12	11	33	16	15
42B/47C	1	1	0	1	1	0	0	1	1
total	43	46	63	24	23	38	33	33	39
rest of group I	9	7	8	8	4	8	11	5	7
total of group I	52	53	72	32	27	45	44	44	46
total of group II	18	25	12	22	15	17	11	11	16
total of group III	15	14	9	21	29	17	11	22	17
83A	3	3	0	2	9	3	11	7	3
miscel. total	5	5	2	6	12	6	11	8	6
NI	3	3	1	1	4	0	0	3	3
NT	11	10	4	15	14	14	22	12	11
Total number of strains	1270	3399	85	863	1000	63	9	242	6932

Only 1 strain from autopsy is included in the survey
No representatives of group IV were found
For an explanation of the subdivision of the clinical sources see Tables 2 and 3
"52/52A/80" comprises strains of phage types 52/80, 52A/80, 52/52A/80, 52/52A/80/81, 52/80/81 etc.

TABLE 3

Strains from Various Human Sources in Relation to Age-Groups (Percentage Distribution)

Age Year of birth	0 1960	10 1959-50	20 1949-40	30 1939-30	40 1929-20	50 1919-10	60 1909-00	70 1899-90	80 1889-80	90 1879-70	X
Clinical source											
Group A	12	17	24	23	20	20	18	14	16	12	19
Group B	43	43	50	47	53	50	50	49	50	54	49
Mastitis	1	0	2	4	2	0	0	0	0	1	1
Group C	37	29	15	17	9	8	6	4	6	10	13
Group D	3	7	5	7	11	17	21	28	22	19	14
Blood [illegible]	0	0	1	0	1	1	1	2	2	1	1
Group I	4	4	3	2	4	4	4	3	4	3	3
Total	100	100	100	100	100	100	100	100	100	100	100
Total number of strains	323	603	828	974	698	771	952	865	695	223	6932

Specimens from spinal fluid *and autopsies* were *below 1 per cent* each
X = percentage of total number of strains
A specified survey of the clinical sources is seen from Table 2
Group A comprises mostly strains from unspecified sources
Group B comprises mostly strains from skin and subcutaneous tissues
Group C comprises mostly strains *from bodily orifices*
Group D comprises mostly strains from excretes
Group I comprises mostly strains from tissues and bodily fluids having *no connection* with the surface of the body

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Percentage Distribution of Phage Patterns and Groups among Strains from Various Clinical Sources

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Phage type group									
80/81	20	21	35	8	5	21	0	17	17
80	3	4	2	1	3	3	0	3	1
81	4	3	5	3	2	2	0	2	3
52/52A/80	15	17	21	10	12	11	33	16	15
42B/47C	1	1	0	1	1	0	0	1	1
total	43	46	63	24	23	38	33	39	39
rest of group I	9	7	8	8	4	8	11	5	7
total of group I	52	53	72	32	27	45	44	44	46
total of group II	18	25	12	22	15	17	11	11	16
total of group III	15	14	9	21	29	17	11	22	17
83A	3	3	0	2	9	3	11	7	3
miscel. total	5	5	2	6	12	6	11	8	6
NI	3	3	1	1	4	0	0	3	3
NT	11	10	4	15	14	14	22	12	11
Total number of strains	1270	3399	85	863	1000	63	9	242	6932

Only 1 strain from autopsy is included in the survey.
No representatives of group IV were found.
For an explanation of the subdivision of the clinical sources see Tables 2 and 3.
52/52A/80 comprises strains of phage types 52/80, 52A/80, 52/52A/80, 52/52A/80/81, 52/80/81 etc.

TABLE 5

Antibiotic Resistance of the Various Phage Types/Groups

Antibiotic / Phage types groups	Penicillin			Streptomycin			Tetracyclines			Chloramphenicol			Erythromycin			Total
	number	res	res %	number	res	res %	number	res	res %	number	res	res %	number	res	res %	
80/81	429	354	83	426	371	87	410	20	5	409	8	2	216	2	1	1169
80	135	107	79	135	121	90	132	4	3	132	1	1	55	0	0	226
81	81	60	74	81	53	66	80	12	15	80	2	3	42	0	0	225
52/52A/80	521	382	73	516	365	71	512	53	10	511	16	3	221	4	2	1045
42B 47C	40	31	78	39	10	26	39	4	10	39	1	3	25	0	0	81
rest of group I	218	62	28	217	22	10	217	5	2	217	3	1	109	2	2	480
3A	200	104	52	197	6	3	197	6	3	196	6	3	84	0	0	393
71	16	9	56	25	1	4	25	1	4	25	0	0	13	0	0	53
rest of group II	328	103	31	323	6	2	325	9	3	325	6	2	177	1	1	681
group III	555	241	43	548	128	23	548	79	14	546	29	5	255	7	3	1188
group IV	2	0	0	2	0	0	2	1	0	2	0	0	2	0	0	5
83A	117	104	89	117	110	94	117	105	90	113	4	4	52	3	6	242
rest of miscel	87	17	19	84	6	7	84	2	2	85	2	2	45	0	0	160
mixed group	104	54	52	104	23	23	104	20	20	104	4	4	47	0	0	208
NT	418	187	61	412	103	25	412	63	15	412	23	6	185	0	0	776
Total	3251	1898	58	3226	1325	41	3204	384	12	3196	105	3.3	1528	19	1.2	6932

another 5 5 per cent may be added to this group if strains from wounds (this designation may comprise cicatrices, accidental wounds and ulceration of the skin) are added In 18 2 per cent of the specimens the origin was unknown (referred to in the table as unknown, pus, swab, pure culture = group A)

Table 3 shows the number of staphylococcal strains isolated from various human sources in relation to age groups The first column indicates the number of strains from children up to 1 year old All the other columns indicate the number of isolates from patients in the age groups 1–10 years, 10–20 years etc

323 specimens from children up to 1 year old seems a remarkably high figure When the other figures in the table are compared, it will be noted that the highest number of strains originated from patients between 20–30 years old About the same number were isolated from patients 50 60 years old

As might be expected strains from the skin are predominant in all age groups For persons under 30 years, specimens from "bodily orifices (group C) come next, for groups over this age, strains from excretes (group D)

Phage Types

Tables 4 and 5 show that ' type 80 ', comprising types 80, 81, 80 81, 52 52A 80 and 42B 47C, is the one most frequently found It is predominant in all diagnostic groups, especially among mastitis strains The excretes are an exception, here strains belonging to group III form the majority In the total material, group III and group II strains represent 18 and 16 per cent respectively

In the total material the "new type" 83A amounts to only 3 per cent Table 6 gives specified information about its occurrence Most frequently it has been isolated from excretes and exudates

11 per cent of the strains were non typable (NT) On the whole, nontypable strains are evenly distributed over the various sources, the only exception being the mastitis strains, the percentage of which is below the average

Antibiotic Resistance Correlated with Phage Types

Only for half of the strains is there any information about resistance to antibiotics [illegible]
nico
T [illegible] of the strains are penicillin-resistant about 40 per cent resistant to streptomycin, and only 14 per cent resistant to tetracyclines resistance to chloramphenicol and erythromycin is very rare (3 per cent and 1 per cent respectively)

Furthermore, it is obvious (Table 5) that there is a certain correlation between phage types and resistance to antibiotics by far the

greatest number of "type 80" strains are resistant to penicillin and streptomycin, very few are resistant to tetracyclines

Type 83A is almost constantly resistant to penicillin, streptomycin and tetracyclines, and the majority of all the strains resistant to these antibiotics belong to type 83A (Table 7).

About half the strains of phage-groups III, NI and NT are penicillin resistant, about 20 per cent are streptomycin-resistant and comparatively many strains are resistant to tetracyclines

Many strains sensitive to all antibiotics are found among representatives of phage groups I ("type 80" excepted) and II However, about 50 per cent of type 3A strains (group II) are penicillin-resistant

TABLE 6
Occurrence of Phage Type 83A

Isolated from	Total number of strains	Phage type 83A total number	per cent
unknown source	56	2	3 6
region stated	439	12	2 7
pus	954	29	3 0
swab	227	3	1 3
abscesses, etc	1300	15	1 2
pustuli etc	430	9	2 1
hands	472	2	0 4
cicatrices	344	22	6 4
wounds	385	25	6 5
combustion	17	1	5 9
eczema	12	1	8 4
eye	119	1	0 8
ear	240	6	2 5
nose	290	4	1 4
mouth	190	2	1 1
vagina	24	1	4 2
larynx, trachea	129	10	7 8
bronchii	43	5	11 2
sputum	516	41	8 0
urine	258	28	10 9
faeces	31	4	12 9
blood	63	2	3 2
spinal fluid	9	1	11 0
viscera	42	3	7 1
exudate	51	7	13 8
fistulae	99	6	6 1
autopsy	1	0	0
total of phage typed strains	6932	242	3 5

Type 83A was not found in any specimen from mastitis sinus osteomyelitis and joint disease cases

Lipase Production

24 per cent of 6,890 strains investigated could not be shown to produce lipase (Fig 1)

TABLE 7

Occurrence of Strains Resistant to Penicillin Streptomycin and Tetracyclines within the Various Phage Types Groups

Phage type group	Number of strains investigated	Number of strains resistant to P + S + T	
		Total	Per cent
80 81	410	19	4.6
80	132	1	0.8
81	80	6	7.4
52 52A/80	512	34	6.6
42B 47C	39	2	5.1
rest of group I	217	2	0.9
total of group I	1390	64	4.6
3A	197	1	0.5
71	25	1	4.0
rest of group II	325	1	0.3
total of group II	547	3	0.6
total of group III	548	59	10.7
total of group IV	2	0	0
83A	117	95	81.1
rest of miscel	84	2	2.3
total of miscel	201	97	48.2
mixed group	104	16	15.4
NT	412	49	11.9
total	3201	288	9.2

P = penicillin S = streptomycin T = tetracyclines

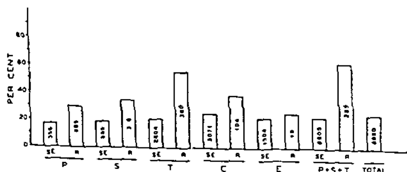


Fig 1

Occurrence of FY negative strains related to antibiotic resistance The figures in the columns indicate number of strains P = penicillin S = streptomycin T = tetracyclines C = chloramphenicol E = erythromycin SE = sensitive R = resistant

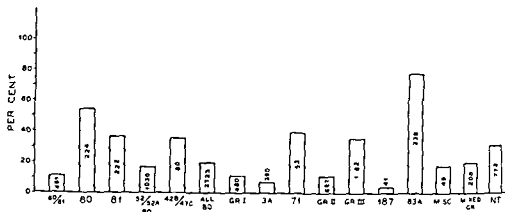


Fig 2

Occurrence of EY negative strains, related to phage types/groups
The figures in the columns indicate number of strains

The occurrence of EY-negative strains was unevenly distributed throughout the material, they were more numerous among antibiotic-resistant strains than among sensitive ones. 61 per cent of staphylococci resistant to penicillin, streptomycin and tetracyclines were EY-negative Thus, the percentage of EY-negative strains also is highest among types with the highest developed resistance to antibiotics (Fig 2) 79 per cent of strains belonging to type 83A are EY-negative "Type 80" taken as a whole is an exception Only 20 per cent of this type are *EY-negative However, in this respect the various representatives within* the type behave differently Types 80 81 and 52/52A/80 have very few EY-negative strains, whereas the other members of the "type" 80, 81 and 42B/47C, are often EY-negative

DISCUSSION

In one respect the present material is unusual It represents a survey of a large number of epidemiologically unrelated phage-typed *Staphylococcus aureus strains collected from about 150 Danish hospitals during* the last nine months of 1960

We have not been able to find any other material directly comparable to the present one Those we have considered all deal with strains collected over several years (*Blair & Carr* 1958) and isolated in a single hospital where it has been possible to make a distinction between hospital cases and community cases (*Bynoe, Elder & Comtois* 1956, *Spink* 1956, *Bauer, Perry & Kirby* 1960, *Wallmark & Finland* 1961) in one respect all these studies disagree with ours in that the resistant strains are less numerous in the Danish material, in particular, strains resistant to tetracyclines, chloramphenicol and erythromycin are seldom encountered by us

"Type 80" which in other parts of the world, too, is the most widespread "type", is rarely reported as being sensitive to tetracyclines,

whereas the Danish representatives of the type are, as a rule, resistant to penicillin and streptomycin only. We do not know whether a more cautious Danish policy as regards the use of antibiotics may account for the divergent antibiotic resistance of the staphylococci suggested by *Bauer, Perry & Kirby* (1960) in a report from an American hospital

Williams (1960) mentions that type 83A has recently become epidemic in England In non-European countries it is not possible at the present moment to follow the trail of this type, because phage 83A has not been widely used for typing We are of the opinion that this new type" is becoming more frequent in Denmark, for in 1961 it caused two minor hospital epidemics As this type combines multi-resistance to antibiotics (to penicillin, streptomycin and tetracyclines) with the possible virulence factor, EY-negativity, it may cause infections difficult to treat and with uncertain outcome Therefore, the wider spread of this type is being closely studied by the present authors

Rather few EY-negative strains are found among representatives of type 80 81 This may probably be explained by the fact that a large percentage of these strains in the present material were isolated from abscesses and furunculi, where EY-positive staphylococci are especially frequently encountered (*Gillespie et al.* 1952, *Jessen et al* 1959a)

During recent years, *Jessen et al* (1959a, 1963) have investigated staphylococci isolated from bacteraemia cases The bacteriological findings of these reports are very similar to those of the present paper, but more penicillin-resistant strains were found among the strains causing bacteraemia A comparison between the two materials is made in another report contained in this issue (*Jessen et al*, p 85)

SUMMARY

On April 1st, 1960, Statens Seruminstitut, Copenhagen, began a registration of all phage-typed *Staphylococcus aureus* strains isolated from persons—patients and staff—connected with Danish hospitals

During the last nine months of 1960, 6,932 persons from about 150 hospitals were registered

This report comprises a survey of one strain—preferably from pathological processes—from these persons It was found that about 50 per cent of the strains were isolated from pathological conditions of the skin and at least 5 per cent from post-operative infections

"Type 80" was the type most frequently found Most of these strains were resistant to penicillin and streptomycin only 3 per cent of the strains belonged to a "new" type, 83A, which was almost constantly resistant to penicillin, streptomycin and tetracyclines 11 per cent were non-typable

Only for half of the strains was there any information about resistance to antibiotics 50 per cent were resistant to penicillin, 40 per cent to streptomycin and 14 per cent to tetracyclines

24 per cent of the strains were lipase-negative (LY-), the percentage being highest among antibiotic-resistant strains

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FROM STATENS SERUMINSTITUT, COPENHAGEN (DEPT. OF DIAGNOSTIC BACTERIOLOGY AND STREPTOCOCCUS DEPT.), AND THE INSTITUTE OF GENERAL PATHOLOGY, COPENHAGEN

SOME PROPERTIES OF STAPHYLOCOCCUS AUREUS, POSSIBLY RELATED TO PATHOGENICITY

Part 3[1] Bacteriological Investigations of Staphylococcus aureus Strains from 462 Cases of Bacteraemia

By

OVE JESSEN, KIRSTEN ROSENDAL, VIGGO FABER, KARL HOVE and KNUD RIEWERTS ERIKSEN

Received 20.xi.62

In two preceding papers (*Jessen, Faber, Rosendal, Eriksen* 1959a, b) an attempt was made to elucidate possible relationships between pathogenicity and some measurable in *vitro* properties of *Staphylococcus aureus*, by correlating the latter with the clinical type and course of infection in a number of human cases (bacteraemia, boils and impetigo lesions)

This approach demands a large material to reduce the influence of many variable, unrecorded or unknown factors For that reason, and in order to detect possible changes in the characters of the prevalent strains, some of the investigations from the years 1957–58, namely those on bacteraemia cases, were continued during the following two years The bacteriological observations in a total of 462 epidemiologically unrelated cases of staphylococcal bacteraemia from these four years are reported here

The [illegible] tality r [illegible] egg yol [illegible] the strains, hospital origin of the infections, and serious primary diseases of the patients No immediate correlation was established between mortality rate and phage type, production of alpha lysin or production of hyaluronidase

The two latter properties were omitted from the present investigations

Previously it had been found (*Jessen, Faber, Rosendal & Eriksen* 1959a) that the strains causing bacteraemia in some respects represented an inhomogeneous group in contrast to those isolated from impetigo lesions and boils In order to see if bacteraemia strains differ from strains from other sources, the present report includes a compa-

[1] Part 1 and 2 Acta path et microbiol scandinav vol 47 p 316 and 327

24 per cent of the strains were lipase-negative (LY-), the percentage being highest among antibiotic-resistant strains

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The two preceding papers indicated a correlation between high mortality rate and the following factors: Antibiotic resistance and negative egg-yolk reaction of the strains, hospital origin of the infections, and serious primary diseases of the patients. No immediate correlation was established between mortality rate and phage type, production of alpha lysin or production of hyaluronidase.

The two latter properties were omitted from the present investigations.

Previously it had been found (*Jessen, Faber, Rosendal & Eriksen* 1959 b) that the strains causing bacteraemia in some respects represented an inhomogeneous group in contrast to those isolated from impetigo lesions and boils. In order to see if bacteraemia strains differ from strains from other sources, the present report includes a compa-

[1] Part I and II: Acta path. et microbiol. Scandinav. 1959, 47: 316 and 327.

rison between bacteraemia strains and a large material of staphylococci as regards phage-type, resistance to antibiotics and occurrence of Ey-negative strains This large material was isolated from specimens from Danish hospitals during the last 9 months of 1960, and a survey is published elsewhere in this issue (*Rosendal, Slenderup, Helms & Eriksen* 1963)

Recently *Moore* (1960) demonstrated a bimodal distribution of *Staphylococcus aureus* strains when they were characterized according to the concentration of mercuric chloride required for complete growth inhibition From concurrent clinical experience he advanced the hypothesis that a relation exists between mercury-resistance and the epidemic character of the strains Because of this observation the mercury resistance of all the bacteraemia strains has been investigated

MATERIAL

The material consists of 497 strains of coagulase positive *Staphylococcus aureus* strains isolated at Statens Seruminstitut and the Blegdamshospital by blood cultures *from 462 patients with bacteraemia This number comprises 201 previously reported* cases from 1957 58 and 261 cases from the years 1959 60 Clinical information in all but 6 cases was obtained from the hospitals concerned[1] The sex and age of the patients are given in a paper dealing with some clinical aspects of the cases (*Rosendal Faber Hone Jessen & Eriksen* 1962) The laboratory examination of a few strains is incomplete in one or several respects which explains minor differences in the total number of strains in the tables

METHODS

Phage typing antibiotic sensitivity tests and egg yolk reaction were performed as described previously (*Jessen Faber Rosendal Eriksen* 1959 a) Resistance to mercuric chloride was examined by the method given by *Moore* (1960) using freshly prepared agar plates containing $HgCl_2$ in a concentration of 1 75000 and inoculated spotwise with one loopful of an 18 hrs broth culture The results were recorded after 48 hours' incubation at 37° C

RESULTS

Frequency of Staphylococcal Bacteraemia

The number of cases diagnosed in each of the four years 1957 60 was 101, 100, 138, and 123 respectively These figures are not assumed to reflect a real increase in the frequency of bacteraemia within the four-year period, but a stimulated interest in bacteriological investigations as the number of blood specimens examined per year increased by 50 per cent during the period For the whole period, however, staphylococcal bacteraemia seems more common than in the preceding four-year period (*Jessen, Faber, Rosendal* 1959)

Hospital infections accounted for 50 per cent of the cases in 1957 the percentage decreased to *43 in 1958, 41 in 1959 and 37 per cent in* 1960

[1] Our thanks are due to numerous hospital departments for placing clinical data at our disposal

Phage-Types

No significant change was recorded in the relative number of the predominating phage-types during the period investigated The most common single types are still 80/81 and 52/52A/80/81, accounting for 47 per cent of the hospital infections and 35 per cent of the cases originating outside hospitals (Table 1) However, the tendency to focus attention exclusively upon these types is not justified, since more than half of the cases were due to other types It is noticed that phage-group II is responsible for only 8 per cent of the hospital infections

TABLE 1

Phage Types and Origin of the Strains

Origin	Total number	Type 80/81 per cent	Type 52/52A/80/81 per cent	Group I other types per cent	Group II per cent	Group III per cent	NI per cent	NT per cent
Hospital	219	32	15	6	8	23	6	10
Community	212	25	10	8	18	24	6	9
Total material	488	28	12	7	13	24	6	10

Phage types of 488 strains isolated from 462 patients Nine strains were not phage typed The total material includes 57 strains of unknown origin

In 1960 phage 83A was included in the set of phages used The occurrence of type 83A in the earlier material was investigated by retesting all strains previously classified as non-typable, and all strains referred to group III or to the group of non identified strains but susceptible to undiluted phage suspensions only In the present material type 83A was isolated during the four years from 2, 4, 2, and 8 patients respectively, from 14 different hospitals Of these 16 cases 13 were known to be hospital acquired, and the origin of the remaining 3 cases was unknown

Antibiotic Resistance

Antibiotic resistance is much more frequent among hospital strains than among strains originating outside hospitals (Fig 1) In both of the two groups a rapidly increasing incidence of strains resistant to penicillin and streptomycin has been recorded since 1953 (*Jessen, Faber, Rosendal & Eriksen* 1959c) but seems to culminate in 1958

The number of tetracycline-resistant stra[illegible]

[illegible] III, NI, Miscellaneous, and NT (Table 2), whereas the types 80/81 and 52/52A/80/81 as a rule are resistant to penicillin and streptomycin, but not to tetracyclines, chloramphenicol or erythromycin

The increase in the number of tetracycline-resistant strains can be partly ascribed to the occurrence of strains of type 83A Out of 16 strains of this type 14 were tetracycline-resistant

Five strains only were resistant to chloramphenicol, and seven to erythromycin

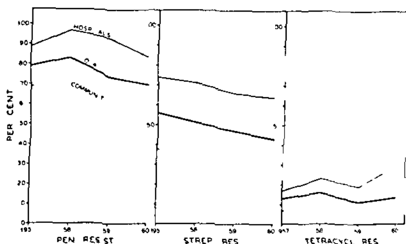


Fig 1

Strains resistant to penicillin streptomycin and tetracyclines in the years 1957 60 (percentage distribution)

TABLE 2

Phage-Types and Antibiotic Resistance

Phage group or type	Number of strains	Penicillin resistant per cent	Strepto mycin resistant per cent	Tetra cycline resistant per cent	Chloram phenicol resist nt	Erythro mycin resistant
80/81	132	97	86	6	0	3 strains
52/52A/80 81	57	89	85	4	2 strains	1 strain
Gr I others	36	58	22	6	0	0
Group II	63	40	3	5	0	0
Group III	113	81	22	29	3 strains	1 strain
NI + Miscel	28	64	32	25	0	1 strain
NT	49	59	39	37	0	1 strain

Antibiotic resistance of 478 bacteraemia strains NT = non typable NI = not identified

Mercury-Resistance

The mercury resistance was investigated in 430 strains The incidence of resistant strains was very constant through the four years 43, 46, 46, and 45 per cent respectively

There is a pronounced *correlation between antibiotic resistance and* mercury-resistance in so far as strains sensitive to all antibiotics practically always were sensitive to mercury as well Among strains resistant to penicillin but sensitive to other antibiotics one fifth were mer-

TABLE 3

Resistance to Mercuric Chloride Related to Phage-Type and Antibiotic Resistance

	Resistant to	O per cent	P only per cent	P+S only per cent	P+S+T only per cent	S only	T only per cent
Hg resistant	80/81 and 52/52A/80/81	0 — (0/8)	81 — (13/16)	85 — (115/135)	86 — (6/7)	(1/1)	(0/0)
	Other types	3 — (2/80)	10 — (10/102)	41 — (9/22)	75 — (24/32)	(0/2)	63 — (5/8)
	Total	2 — (2/88)	20 — (23/118)	80 — (124/157)	77 — (30/39)	(1/3)	63 — (5/8)

P — penicillin S — streptomycin T — tetracyclines

The numerator indicates number of Hg resistant strains, the denominator indicates the total number of strains

cury resistant, and among those resistant to penicillin and streptomycin four fifths were resistant (Table 3)

However, this concordance between resistance to antibiotics and Hg resistance to a certain extent reflects another correlation, viz between Hg resistance and phage-type If strains having identical resistance are considered it is evident from Tables 3 and 4 that certain phage-types are mercury-resistant far more often than others with the same antibiotic resistance It is most easily seen for the types 80/81 and 52/52A/80/81 (Table 3) and for type 83A (Table 4)

TABLE 4
Proportion of Hg Resistant Strains in Different Phage Groups

Phage type or group	P sensitive	P resistant			
		Total		Resistant to P and S	
	Number	Number	Per cent	Number	Per cent
80/81	1/3	97/114	85	83/99	84
52/52A/80/81	0/6	39/46	85	38/43	88
Gr I others	0/14	7/21	33	6/8	75
Group II	*1/32*	*1/24*	*4*	*0/0*	
Type 83A	0/0	13/15	87	13/13	100
Gr III others	4/21	16/77	21	4/16	25
NI + Miscel	1/8	5/14	36	1/4	25
NT	1/15	9/20	59	9/11	82
Total	8/99 = 8%	187/331	58	154/194	79

A total of 430 strains investigated for Hg resistance In this table type 83A strains have been removed from the groups III NI and NT and listed separately
P = penicillin S = streptomycin NT = non typable NI = not identified

Mercury-resistance was more frequent in hospital strains (58 per cent) than in community strains (36 per cent) This distribution was to be expected because of the predominance of antibiotic resistant strains and of strains of phage type "80" and 83A in hospitals The difference is greatly reduced if only penicillin resistant strains are considered (Hospital 59 per cent, community 54 per cent) and it disappears entirely if strains of the same phage type are compared Thus, the difference in Hg reaction between hospital and community strains may be entirely secondary to these two other factors

Correspondingly, no direct correlation could be established between Hg resistance on one side, and on the other side the portal of entry of the infections, the egg yolk reaction of the strains or in 150 strains investigated—the amount of hyaluronidase or of alpha lysin produced

Egg-Yolk Reaction

The frequency and distribution of the EY-negative strains corresponded to that found previously (*Jessen, Faber, Rosendal & Eriksen*

1959a) The negative EY-reaction was most common among penicillin- and streptomycin resistant strains of phage-group I and III, and among hospital strains

Bacteraemia Strains Compared with 6932 Strains from Miscellaneous Sources

The latter strains have been isolated from a variety of clinical sources including skin, noses etc (*Rosendal, Stenderup, Helms & Eriksen* 1963) during 1960 As they have been collected from about 150 hospitals all over Denmark, they have been considered epidemiologically unrelated

TABLE 5

Phage Types of Bacteraemia Strains as Compared with a Material of Strains from Other Sources

		80/81 per cent	52/52A/80/81 per cent	Group I others per cent	Group II per cent	Group III per cent	NT per cent	83A per cent	VI + miscellaneous per cent
Bacteraemia	488 strains	28	12	7	13	24	11	3	3
Miscellaneous	6932 strains	23	15	7	16	17	11	3	6

From Table 5 it is seen that the phage-types of the two materials correspond closely to each other The same is true of resistance to streptomycin and tetracyclines, whereas the miscellaneous material comprises a relatively greater number of penicillin-sensitive strains than does the bacteraemia material, also when strains exclusively from 1960 are compared (Table 6)

EY-negative strains are encountered with the same frequency in both materials, about 25 per cent in each

TABLE 6

Antibiotic Resistance of Bacteraemia Strains as Compared with a Material of Strains from Other Sources

Resistant to	Penicillin	Streptomycin	Tetracyclines
	Per cent	Per cent	Per cent
Bacteraemia total (488 strains)	76	49	12
Bacteraemia 1960 (120 strains)	70	43	13
Miscellaneous 1960 (3251 strains)	58	11	12

Mortality Rate Dependence on Host Factors and Origin of the Infection

Mortality rate was slightly lower in 1958 60 than in 1957 (Table 7) in all groups of patients, i e irrespective of the origin of the infection,

TABLE 7

Mortality through 4 Years

	1957	1958	1959	1960	Total
	Per cent	Per cent	Per cent	Per cent	Per cent
Hospital	59	51	52	51	53
Community	35	22	27	26	27
Total, incl unknown origin	48	35	39	35	39

the antibiotic resistance of the strains, and other factors considered

About one fifth of the patients infected outside hospitals, and two fifths of the hospital-infected patients were considered especially prone to develop serious infections because of primary diseases such as neoplasms, severe blood disorders, cirrhosis of the liver, diabetes, and severe renal failure The overall mortality rate in this group of patients was 54 per cent as against 33 per cent for patients without these complications

The group of hospital-infected patients comprises many of these bad risks; however, this fact does not fully explain the great difference in mortality rate between the hospital group and the community group (Table 8) as the difference is especially pronounced in patients without serious complications

TABLE 8

Serious Pre Existing Conditions Influencing Mortality

Origin	Complicating conditions present		No pre existing complications		Total mortality per cent
	Number	Mortality per cent	Number	Mortality per cent	
Hospital	81	60	114	48	53
Community	40	53	174	21	27
Total incl unknown origin	137	54	320	31	39

Mortality Rate Relation to Antibiotic Resistance

In the following sections cases due to more than one strain are omitted The figures referred to are given in Table 9

The mortality rate for different groups of patients varied from 21 per cent for those infected outside hospitals with penicillin sensitive strains to 67 per cent for patients infected with streptomycin-resistant —and that is, practically always, also penicillin resistant hospital strains

For patients infected with penicillin- and streptomycin-sensitive strains the origin of the infection was of minor importance for the prognosis, but for infections due to resistant strains the above-mentioned difference between hospital and non-hospital strains is striking

The total material shows a mortality rate for infections due to penicillin- or streptomycin-resistant strains more than twice as high as that for infections due to sensitive strains

TABLE 9

Antibiotic Resistance of the Infecting Strains Correlated to Mortality

Origin	Penicillin		Streptomycin		Tetracyclines	
	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Hospital	59 (162)	24 (17)	67 (123)	30 (56)	65 (37)	54 (132)
Community	31 (127)	21 (76)	34 (64)	24 (139)	33 (9)	27 (194)
Total incl unknown	46 (321)	22 (104)	55 (209)	24 (216)	54 (50)	39 (375)

Mortality in 42> cases divided into groups according to the origin of the infection and the antibiotic resistance of the strain Mortality is *given in per cent The number* of cases in each group is indicated in brackets In the total group is included 42 cases of unstated origin

Mortality Rate Relation to EY Reaction

Infections due to EY- and EY + strains had a mortality rate of 58 and 38 per cent respectively However, the proportion of EY– strains is not uniform in all groups of the material It is highest in hospital strains resistant to penicillin and streptomycin Therefore a possible relation between mortality and EY reaction must be evaluated in groups where the strains are of uniform origin and antibiotic resistance Other factors influencing mortality, such as the presence of complications, the age of the patients, and the portal of entry of the infections, could be shown not to influence the conclusions, neither could they be expected to do so as the proportion of EY– strains was fairly uniform in groups established along these lines of division

TABLE 10

Mortality and EY Reaction in Comparable Groups of Patients

	Hospital		Community		Hospital		Community	
	P sens	P res	P sens	P res	S sens	S res	S sens	S res
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
EY	29 (14)	56 (95)	20 (56)	26 (97)	29 (38)	65 (71)	20 (104)	33 (49)
EY	[illegible] (8)	67 (63)	30 (13)	52 (21)	46 (13)	72 (50)	39 (23)	45 (11)

Percentages indicate mortality rate
Total number of cases in each group is given in brackets

The required comparison is carried out in Table 10 The splitting up of the material into eight groups makes the figures small, and very considerable differences in mortality are required to obtain statistically significant results However, the results are sufficient to call for further

investigations of possible fundamental differences between EY- and EY+ strains In each of the eight groups established in Table 10 mortality rate is highest for infections due to EY– strains Furthermore, for the groups of community infections the mortality rates increase through the following groups EY+ sensitive, EY+ resistant, EY–sensitive EY– resistant For the hospital infections the group of sensitive EY– strains is small or non-existent

In the total material 40 per cent of 211 patients infected with EY+ penicillin resistant strains died, whereas the mortality rate among 95 patients infected with EY- penicillin resistant strains was 62 per cent

Mortality Rate Relation to Phage-Type

An overall estimation will charge infections due to certain phage types, *e g* 80/81, with a higher mortality rate, and infections due to other types, *e g* group II strains, with a mortality rate lower than the average (Table 11, last column) This does not necessarily imply that *e g* a type 80/81 strain *per se* is more virulent in the clinical situation investigated than any other strain The difference seems to reflect two more important factors The prevalence of type 80/81 strains in the hospital infection group, and of group II strains in the community group, and the generally higher antibiotic resistance of the former type of strains, which might have caused therapeutic failure in a great number of cases

TABLE 11
Mortality and Phage Type of the Infecting Strains

Origin of infection	Hospital		Community		Hospital+ Community +unknown
Penicillin sensitivity	Resistant	Sensitive	Resistant	Sensitive	Resistant+ sensitive
	Per cent	Per cent	Per cent	Per cent	Per cent
80/81	66 (62)	0 (2)	32 (46)	0 (2)	49 (131)
52/52A/80/81	57 (30)	0 (2)	31 (16)	25 (4)	44 (55)
Gr I others	70 (10)	100 (1)	43 (7)	11 (9)	44 (32)
Group II	0 (6)	40 (5)	17 (12)	20 (24)	19 (53)
Group III	55 (29)	20 (5)	33 (33)	15 (13)	39 (92)
NI + miscel	67 (9)	(0)	0 (6)	17 (6)	36 (22)
NT	56 (16)	0 (1)	50 (4)	36 (14)	41 (41)

Mortality rate is given in percentages Total number in each group is given in brackets

It is convincingly shown in Table 11 that when the origin of the infection, and the antibiotic resistance of the strains are taken into consideration, no difference in mortality rate can be said to depend upon difference in the phage-type of the infecting organism

Mortality Rate Relation to Mercury Resistance

The present material confirms the observation (*Moore 1960*) that mercury resistance is especially characteristic for antibiotic resistant strains common in hospitals and belonging to certain phage types known for their epidemic occurrence. Some of the possible explanations have been discussed by *Moore*. One possibility is a difference in pathogenicity between mercury resistant and sensitive strains causing a selection of the mercury resistant strains in hospitals. Although the present material permits an evaluation of a small facet of pathogenicity only, the property was included in the investigations.

Regarding the material as a whole, mortality rate for infections due to Hg resistant strains is 50 per cent as against 33 per cent for infections with Hg sensitive strains. This difference reflects the preponderance of antibiotic resistant strains in the group of Hg resistant strains as shown in Table 3. From the table it appears that practically no antibiotic sensitive strains were Hg resistant.

TABLE 12

Mortality Related to Mercury Resistance of the Strains

	Origin	Hg resistant	Hg-sensitive
		Per cent	Per cent
1 Total material	not considered	50 (182)	33 (201)
Penicillin sensitive	not considered	0 (5)	23 (86)
Penicillin resistant	not considered	51 (177)	41 (115)
3 Penicillin resistant	Hospital	65 (96)	52 (54)
Penicillin resistant	Community	33 (64)	29 (52)
4 Streptomycin sensitive	Hospital	30 (30)	27 (165)
Streptomycin resistant	Hospital	54 (152)	56 (36)

The figures in brackets indicate total number of strains investigated

When the material is split up according to penicillin resistance (Table 12 2) the difference in mortality rate is greatly reduced and found only in the group of infections due to penicillin resistant strains. In part 3 of the table the difference is referred to the penicillin resistant hospital strains but when the groups of hospital strains are divided according to streptomycin resistance mortality rate is unrelated to the Hg resistance of the strains (part 4).

DISCUSSION

Any relation between biochemical properties of micro organisms and their pathogenicity for man must be confirmed [illegible] ions of [illegible] This is [illegible] which is [illegible]

In the present study it is attempted to avoid the bias of clinical evaluation of the severity of the infections by defining strictly the facts observed and the conditions studied. The measure of pathogenicity is the death or survival of the patient in the situation where staphylococci have been demonstrated to gain entrance to the bloodstream.

As a consequence the material comprises cases of acute fulminating septicaemia almost sure to run a fatal course, as well as cases of bacteraemia with less serious symptoms. This is no inconvenience, but a crucial point in the study, the very aim of which is to elucidate whether such differences are determined largely by host factors such as age, primary diseases, portal of entry etc., or whether they can be correlated to measurable bacterial properties.

A common source of error has been avoided by collecting the material from the whole country so that no immediate epidemiological relation exists between the strains.

Nevertheless the method is open to criticism. The numerous factors which are scrupulously considered and standardized in animal experiments are to a great extent unknown or variable here. By means of the clinical information and by dividing the material along many different lines we have tried to point out some of the important factors influencing prognosis and explain their interpendence. These factors include the origin of the infection, *i.e.* whether the infection was contracted in or outside hospital, the presence of serious primary diseases, and the antibiotic resistance of the strains.

The last-mentioned factor can be assumed to influence mortality primarily by reducing the chances of effective antibiotic treatment. The question whether the antibiotic-resistant strains beyond this *per se* are more virulent than the sensitive ones can be answered only by examining a material of untreated cases. Comparison between about 3000 miscellaneous strains and the bacteraemia strains does not indicate that the strains causing bacteraemia represent a selection of the resistant strains.

The confirmation of the connection between negative EY reaction and high mortality suggests that further work on the nature of this reaction and its connection with other biochemical or physical properties *might provide valuable information.* Because of the considerable number and the serious character of the infections due to EY-negative strains the proposal to limit phage-typing to EY positive strains in epidemiological investigations (*Graber et al.* 1958) must be rejected. In *any case the use of this character in the selection of strains on primary* isolation (*Carter* 1960) is unfortunate, especially as we have recorded some hospital epidemics due to EY-negative strains. Investigations of strains in serial subcultures and of 25 colonies of each of 109 bacteraemia strains, *and of strains isolated successively from the same patient* have indicated that the reaction is sufficiently stable to be of use in epidemiological investigations. It is of special use in the analysis of

epidemics due to very common types where the phage type alone is not sufficient indication of epidemiological connection

Supplementary epidemiological information may be provided by the *Hg reaction* as it is independent of the EY reaction and is regarded as reasonably constant. Our investigations confirm *Moore's* (1960) observation of a bimodal distribution of the strains and confirm that the majority of the antibiotic resistant strains of phage types 80/81, 52/52A/80/81, 83A and certain other types are Hg resistant. The present material cannot provide evidence in favour of or against *Moore's* suggestion of a connection between Hg resistance and epidemic occurrence. As an exception *Moore* reports two outbreaks due to Hg sensitive strains. In Denmark one similar outbreak was recorded. All of the three epidemics occurred in maternity wards. The lack of correlation between the Hg reaction and mortality, production of alpha lysin and hyaluronidase and the EY reaction does not mean that the Hg reaction may not reflect an important metabolic or structural property of the strains.

The *phage sensitivity* of the strains is an example of a property related to some facets of pathogenicity and not to others. Type 71 strains have been demonstrated to be typical for impetigo lesions and cutaneous boils are most often caused by group I strains, but in an established bacteraemic condition phage sensitivity cannot be shown to influence the final outcome of the infection. Neither are certain types more likely to cause bacteraemia than others, as phage types of the bacteraemia material correspond closely to those of about 7000 strains [illegible]

[illegible] cases of bacteraemia caused by type 83A strains. The cases occurred in 14 different hospitals where there was no probable epidemiological connection. Therefore the almost constant connection between this phage type and resistance to penicillin, streptomycin and tetracyclines, negative EY reaction and Hg resistance may be a characteristic of the phage-type. The type has been uncommon, but is encountered with increasing frequency. It made up 3 per cent of about 7000 strains from 1960 and 16 bacteraemia cases in 1961 were caused by type 83A. Its occurrence will be followed with attention.

CONCLUSIONS AND SUMMARY

When bacteraemia has been established the [illegible]

year period [illegible]

a *number of bacterial* characters and mortality, and to investigate to what extent mortality rate was influenced by other known factors

The total mortality rate was 39 per cent Prognosis was greatly influenced by the coexistence of serious primary diseases, and mortality rate was higher in hospital infections than in infections contracted outside hospitals Infections due to penicillin- and streptomycin-resistant strains have a mortality rate about twice that for infections due to sensitive strains Bacteraemia due to EY-negative strains is more often fatal than cases due *to infection with EY-positive strains The EY reaction* may be of some value in epidemiological investigations

Mercury-resistance is not primarily related to mortality but is intimately correlated to resistance to penicillin and streptomycin, and is most common in strains of the phage-types 80/81, 52 52A/80 81 and 83A

When the *influence on mortality of the origin of the infection*, the antibiotic resistance of the strains etc are eliminated, the phage type of the strains is unrelated to mortality Recently, several serious infections due to phage-type 83A were recorded It is suggested that the characteristic properties of these strains may favour their future epidemic occurrence

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AUS STATENS SERUMINSTITUT
(INTERNATIONALE SALMONELLA CENTRALE) KOPENHAGEN

ZUR SEROLOGIE DER SALMONELLA O-GRUPPEN
30, 42, 43, 48 und 50

Von

F. KAUFFMANN und A. PETERSEN

Eingegangen 30. xi. 62

In Fortsetzung einer vorhergehenden Mitteilung von *F. Kauffmann & A. Petersen* „*Zur Serologie der Salmonella O-Gruppen 28, 40, 45 und 47*" berichten wir im folgenden über Untersuchungen der O-Gruppen 30, 42, 43, 48 und 50. Da auch diese O Gruppen komplex gebaut sind, so wurde hierauf näher eingegangen, zumal diese Verhältnisse nicht allgemein bekannt sind. Ferner wurden im Laufe der letzten Jahre zahlreiche *species* dieser O Gruppen gefunden, ohne dass in allen Fällen eine Einordnung in die betreffenden O-Untergruppen vorgenommen wurde. Es sei jedoch betont, dass diese O Gruppen im *Kauffmann-White-Schema* nach wie vor mit 30, 42, 43, 48 und 50 bezeichnet werden, sodass also die O Untergruppen hierin nicht berücksichtigt werden.

Die O Gruppe 30

Wie *F. Kauffmann & K. Boore* mitteilten, ist das O Antigen von *S. soerenga* = 30 i. 1,w von dem O Antigene des Test-Stammes der O-Gruppe 30 *S. urbana* verschieden. *S. soerenga* war nicht in der Lage, das O Serum von *S. urbana* völlig zu erschöpfen, da Agglutinine für *S. urbana* im Serum zurückblieben. Andererseits wurde aber das O-Serum von *S. soerenga* durch *S. urbana* restlos erschöpft. Die gegenseitigen Antigenbeziehungen können daher folgendermassen ausgedrückt werden:

$$S.\ urbana = 30_1, 30_2$$
$$S.\ soerenga = 30_1$$

Durch Absorption des O-Serum von *S. urbana* mit *S. soerenga* wurde das 30_2-Faktor-Serum hergestellt und eine Untersuchung aller bekannten *species* der O-Gruppe 30 hierin vorgenommen. Es zeigte sich ...
S. ... [illegible]
ub... [illegible]
ur... [illegible] ... Antigenstruktur 30_1

7* [illegible]

a number of bacterial characters and mortality, and to investigate to what extent mortality rate was influenced by other known factors

The total mortality rate was 39 per cent Prognosis was greatly influenced by the coexistence of serious primary diseases, and mortality rate was higher in hospital infections than in infections contracted outside hospitals Infections due to penicillin- and streptomycin resistant strains have a mortality rate about twice that for infections due to sensitive strains Bacteraemia due to EY-negative strains is more often fatal than cases due to infection with EY-positive strains The EY reaction may be of some value in epidemiological investigations

Mercury-resistance is not primarily related to mortality but is intimately correlated to resistance to penicillin and streptomycin, and is most common in strains of the phage-types 80 81, 52/52A/80 81 and 83A

When the influence on mortality of the origin of the infection, the antibiotic resistance of the strains etc are eliminated, the phage type of the strains is unrelated to mortality Recently, several serious infections due to phage-type 83A were recorded It is suggested that the characteristic properties of these strains may favour their future epidemic occurrence

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Die O Gruppe 43 kann in 3 O Untergruppen eingeteilt werden

43_1 43_2, 43_3 = *S milwaukee*

43_1, 43_3 = *S kingabwa*, *S ahuza*, *S berkeley*, *S farcha*, *S irigny*, *S mbao* und *S tuindorp*

43_1, 43_3, 43_4 = *S bunnik*, *S houten*, Arizona 21 und *Escherichia* 86 B7

Um zu einer Vereinfachung der Antigenformeln zu gelangen, kann man den Faktor 43_3 fortlassen, sodass man nur 2 verschiedene O Untergruppen unterscheidet

43_1 43_2 = *S ahuza*, *S berkeley*, *S farcha*, *S irigny*, *S kingabwa*, *S mbao*, *S milwaukee* und *S tuindorp*

43_1 43_4 = *S bunnik*, *S houten*, Arizona 21 und *Escherichia* 86 B7

Mit Hilfe von 2 Faktor-Seren 43_2 und 43_4 ist es bereits in der Objektglas Agglutination möglich, die Diagnose zu stellen

Kultur	Serum 43_2	Serum 43_4
43_1 43_2	++	—
43_1 43_4	—	++

In der Tabelle 1 sind einige der ausgeführten Absorptions-Versuche, aus denen die obigen Formeln abgeleitet wurden, wiedergegeben

Die zur Differentialdiagnose der O Untergruppen nötigen Faktor-Seren können wie folgt hergestellt werden

43_2-Serum = O Serum von *S milwaukee* absorbiert mit Arizona 21

43_4 Serum = O Serum von Arizona 21 absorbiert mit *S milwaukee*

Anstelle von Arizona 21 kann man auch *S bunnik* oder *S houten* benutzen

Weitere, nicht in der Tabelle 1 angegebene Absorptionsversuche zeigten, dass das Arizona 21 Serum von *S bunnik* oder *S houten* völlig erschöpft wurde Ferner wurde durch gekreuzte Absorption die O Identität von *S bunnik* und *S houten* festgestellt

Wie schon seit langem bekannt, sind Arizona O 21 und *E coli* 86 hinsichtlich ihrer O Antigene sehr nahe verwandt, doch nicht völlig identisch Praktisch gesehen, kann man sie aber als identisch betrachten und zur O Untergruppe 43_1 43_3, 43_4 rechnen

Im originalen *Kauffmann White Schema* werden aber nach wie vor alle Mitglieder der O 43 Gruppe nur mit 43 bezeichnet und durch ein polyvalentes Serum das mit *S milwaukee* + *S bunnik* oder Arizona 21 hergestellt ist diagnostiziert

Die O Gruppe 48

Diese O Gruppe kann in 2 O Untergruppen eingeteilt werden

48_1 48_2 *S dahlem* *S hammonia* *S ngozi* und *S sakaraha*

48_1 48_2 48_3 = *S djakarta* und *S isingen*

Ferner gehören zu dieser O Gruppe 48 die *S arizonae species* 5 17 20 = 48 z₃₆ sowie eine neue von *Dr Cohen*, Sofia, erhaltene *S*

S angoda, S aqua, S bodjonegoro, S donna, S gege, S kumasi, S landau, S matopeni, S morehead, S morocco, S odozi, S ramat-gan und *S zehlendorf.*

Die O-Gruppe 42

In der entsprechenden Weise wie in der O Gruppe 30 war auch in der O-Gruppe 42 von *F Kauffmann & K Bovre* festgestellt worden, dass *S loenga* = 1,42 · z_{10} z_6 hinsichtlich des O-Antigenes von dem Test Stamme dieser Gruppe *S weslaco* verschieden war Während das O Serum von *S weslaco* durch *S loenga* völlig erschöpft wurde, konnten *S weslaco + S senftenberg* das O-Serum von *S loenga* nicht restlos absorbieren Es blieben Agglutinine für *S loenga* im Serum zurück Die gegenseitigen Antigenbeziehungen können daher folgendermassen ausgedrückt werden.

S weslaco = 42_1
S loenga = 1,42_1, 42_2

Durch Absorption des O-Serum von *S loenga* mit *S weslaco + S senftenberg* wurde das 42_2 Faktor-Serum hergestellt und eine Prüfung aller bekannten *species* der O-Gruppe 42 hierin ausgeführt Es zeigte sich, dass folgende *species* das 42_2-Antigen besassen *S egusitoo, S harvestehude, S kampala, S kaneshie* und *S maricopa* Da alle diese *species* das O 1-Antigen enthalten, so ist dieser Faktor 42_2 wahrscheinlich irgendwie an das O 1-Antigen gekoppelt, doch wurde *S paratyphi A* nicht vom 42_2-Serum agglutiniert Ebenso wie aber das O 1-Antigen den O 1-Formenwechsel aufweist, liegt auch ein 42_2-Formenwechsel vor Alle Kolonien, die das O 1-Antigen stark entwickelt haben, besitzen auch ein starkes 42_2-Antigen, während umgekehrt alle Kolonien, welche das O 1-Antigen nur sehr schwach entwickelt haben, auch nur sehr wenig 42_2-Antigen enthalten

Die folgenden *species* besassen weder das O 1-Antigen noch den 42_2-Faktor *S chinovum, S detroit, S fremantle, S nairobi, S portbech, S rand* und *S uphill* Sie enthielten also nur das 42_1-Antigen

Die O-Gruppe 43

Im Jahre 1954 teilten *F Kauffmann & J Vandepitte* mit, dass eine neue *Salmonella species S kingabwa* ein von *S milwaukee*, dem Test-Stamme der O-Gruppe 43, abweichendes O-Antigen habe Das O-Serum von *S milwaukee* konnte nicht durch *S kingabwa* völlig absorbiert werden, es blieb ein Sonderfaktor für *S milwaukee* im Serum zurück

Da dieser Sonderfaktor bisher nur bei *S milwaukee* nachgewiesen wurde, so haben wir jetzt untersucht, bei welchen Kulturen der O-Gruppe 43 dieser Faktor vorkommt Wie es aus folgender Aufstellung hervorgeht, kommt er auch bei *S bunnik, S houten*, Arizona O 21 und *Escherichia* 86 B7 vor

Die O Gruppe 43 kann in 3 O Untergruppen eingeteilt werden

43_1, 43_2, 43_3 = *S milwaukee*

43_1, 43_2 = *S kingabwa*, *S ahuza*, *S berkeley*, *S farcha*, *S irigny*, *S mbao* und *S tuindorp*

43_1 43_3, 43_4 = *S bunnik*, *S houten*, Arizona 21 und *Escherichia* 86 B7

Um zu einer Vereinfachung der Antigenformeln zu gelangen, kann man den Faktor 43_3 fortlassen, sodass man nur 2 verschiedene O Untergruppen unterscheidet

43_1 43_2 = *S ahuza*, *S berkeley*, *S farcha*, *S irigny*, *S kingabwa*, *S mbao*, *S milwaukee* und *S tuindorp*

43_1 43_4 = *S bunnik*, *S houten*, Arizona 21 und *Escherichia* 86 B7

Mit Hilfe von 2 Faktor Seren 43_2 und 43_4 ist es bereits in der Objektglas Agglutination möglich die Diagnose zu stellen

Kultur	Serum 43_2	Serum 43_4
43_1 43_2	++	—
43_1 43_4	—	++

In der Tabelle 1 sind einige der ausgeführten Absorptions Versuche, aus denen die obigen Formeln abgeleitet wurden, wiedergegeben

Die zur Differentialdiagnose der O Untergruppen nötigen Faktor Seren können wie folgt hergestellt werden

43_2-Serum = O Serum von *S milwaukee* absorbiert mit Arizona 21

43_4 Serum = O Serum von Arizona 21 absorbiert mit *S milwaukee* Anstelle von Arizona 21 kann man auch *S bunnik* oder *S houten* benutzen

Weitere, nicht in der Tabelle 1 angegebene Absorptionsversuche zeigten, dass das Arizona 21 Serum von *S bunnik* oder *S houten* völlig erschöpft wurde Ferner wurde durch gekreuzte Absorption die O Identität von *S bunnik* und *S houten* festgestellt

Wie schon seit langem bekannt, sind Arizona O 21 und *E coli* 86 hinsichtlich ihrer O Antigene sehr nahe verwandt, doch nicht völlig identisch Praktisch gesehen, kann man sie aber als identisch betrachten und zur O Untergruppe 43_1, 43_3, 43_4 rechnen

Im originalen *Kauffmann White-Schema* werden aber nach wie vor alle Mitglieder der O 43 Gruppe nur mit 43 bezeichnet und durch ein polyvalentes Serum, das mit *S milwaukee* + *S bunnik* oder Arizona 21 hergestellt ist, diagnostiziert

Die O Gruppe 48

Diese O Gruppe kann in 2 O Untergruppen eingeteilt werden

48_1 48_2 = *S dahlem*, *S hammonia*, *S ngozi* und *S sakaraha*

48_1, 48_2, 48_3 = *S djakarta* und *S hisingen*

Ferner gehören zu dieser O Gruppe 48 die *S arizonae-species* 5 17,20 – = 48 z_{36} – sowie eine neue von *Dr Cohen*, Sofia, erhaltene *S*

TABELLE 1
O Gruppe 43

S. *milwaukee* O Serum

Kultur	Nicht absorb.	Absorbiert mit	
		S. kingabwa	Arizona 21
S. milwaukee	512	256	256
S. kingabwa	512	0	256
Arizona 21	64	8	0
E. coli 86 B7	64	16	0
Faktoren	43_1 43_2 43_3	43_3	43

S. *kingabwa* O Serum

Kultur	Nicht absorb.	Absorbiert mit	
		S. milwaukee	Arizona 21
S. milwaukee	512	0	256
S. kingabwa	512	0	512
Arizona 21	64	0	0
E. coli 86 B7	32	0	0
Faktoren	43_1 43		43_2

Arizona O 21 Serum

Kultur	Nicht absorb.	Absorbiert mit	
		S. milwaukee	S. kingabwa
S. milwaukee	512	0	128
S. kingabwa	512	0	0
Arizona 21	512	128	128
E. coli 86 B7	256	128	128
Faktoren	43_1 43_3 43_4	43_4	43_3 43_4

0 = negativ in 1:4

arizonae species = 48: k: e, n, die ein abweichendes O Antigen besitzt. Der Test-Stamm der Arizona O 5-Gruppe (= Sal. 48: z_3 ...) hat die O Antigene 48_1, 48 , 48_3 und ist hinsichtlich seiner O Antigene mit *S. djakarta* identisch. Die neue *species* 48: k: e,n wurde bisher noch nicht näher untersucht.

Schliesslich gehören zur O 48-Gruppe die atypischen *Citrobacter* Stämme 2624/36 und 5396/38 = Sal. 48, Vi: – 1,5, welche die O Antigene 48_1, 48_3, 48_4 enthalten.

Bei diesen atypischen Citrobacter-Stämmen handelt es sich um Kulturen, die früher mit „*Salmonella coli*“ bezeichnet wurden. Sie nehmen tatsächlich eine Zwischenstellung zwischen *Salmonella*, *E. coli* und *Citrobacter* ein und sind wahrscheinlich Hybriden. Da sie biochemisch am nächsten dem *genus Citrobacter* stehen, obwohl sie Indol bilden und sich hierin wie *E. coli* verhalten, so haben *Erber & Kauffmann* sie atypische *Citrobacter*-Stämme genannt. Sie sind nämlich KCN-positiv, bilden H_2S und sind in Ammonium Citrat positiv. Von *Salmonella* unterscheiden sie sich durch den positiven KCN-Test, die Indol Bildung und die Lactose-Spaltung. Sie stehen serologisch dem *genus Salmonella* sehr nahe, da sie zur O-Gruppe 48 gehören, das Vi Antigen und die H-Antigene 1,5 besitzen.

Das O-Antigen dieser Kulturen war früher mit 31 bezeichnet, wurde aber später aus dem *Salmonella* Schema ausgeschlossen. *Erber & Kauffmann* wiesen nach, dass *S. djakarta* = 31 : z_4, z_{24} : – und *Citrobacter* 2624/36 nahe verwandte O-Antigene besitzen.

TABELLE 2

O Gruppe 48

Kultur	O-Seren			
	S. dahlem		S. djakarta	
	Nicht absorb.	Absorb. mit Citr. 2624/36	Nicht absorb.	Absorb. mit S. dahlem
S. dahlem	640	512	320	0
S. djakarta	1280	1024	1280	512
Ar. 48 : z_{36}	80	128	320	128
Citr. 2624/36	10	0	80	64
Faktoren	48_1 48_2	48_2	48_1 48_2 48_3	48_3

Kultur	Citrobacter 2624/36			
	Nicht absorb.	Absorbiert mit		
		S. djakarta	S. dahlem	S. dahlem + Ar. 48
S. dahlem	20	0	0	0
S. djakarta	640	0	1024	0
Ar. 48 : z_{36}	40	0	128	0
Citr. 2624/36	640	256	512	256
Faktoren	48_1 48_3 48_4	48_1	48_3 48_4	48_4

Die nicht absorbierten Seren wurden ab 1 : 10 angesetzt, die absorbierten Seren ab 1 : 4, da sie in der Verdünnung 1 : 2 absorbiert wurden. 0 = negativ in 1 : 4.
Die O-Identität von *S. djakarta* und Arizona 48 : z_{36} wurde durch gekreuzte Absorptionsversuche nachgewiesen.

Im Supplement II zum *Kauffmann-White-Schema* hat *Kauffmann* mitgeteilt, dass die O-Gruppen 31 und 48 zu einer einzigen O Gruppe 48 vereint wurden, da *S djakarta* – wie *J Taylor* nachwies – mit *S dahlem* nahe verwandt ist Die O-Gruppe 31 wurde gestrichen, da *S djakarta* nicht vollständig glatt war

Der Test-Stamm der O-Gruppe 48, *S dahlem*, wurde von *Kauffmann, Hofmann & Platz* beschrieben Die Autoren teilten mit, dass das O Serum von *S dahlem* durch Arizona O 5 völlig absorbiert wurde, dass aber *S dahlem* nicht imstande sei, das O-Serum von Arizona O 5 zu erschöpfen

Die O-Antigenbeziehungen zwischen *Salmonella* 48, Arizona 5 und den atypischen *Citrobacter*-Stämmen waren also schon bekannt und sind in dieser Arbeit nur näher analysiert worden

Die verschiedenen Kulturen verteilen sich auf die einzelnen O Untergruppen wie folgt.

48_1, 48_2 = *S dahlem, S hammonia, S ngozi* und *S sakaraha*

48_1, 48_2, 48_3 = *S djakarta, S hisingen* und Arizona 48

48_1, 48_3, 48_4 = *Citrobacter* 2624/36 und 5396 38

Zur Differentialdiagnose gebraucht man also 3 Faktor-Sera

48_2 = O-Serum von *S dahlem* oder von *S djakarta* absorbiert mit *Citrobacter 2624/36*

48_3 = O-Serum von *S djakarta* oder von Arizona 48 absorbiert mit *S dahlem*

48_4 = O-Serum von *Citrobacter* 2624/36 absorbiert mit *S djakarta* oder Arizona 48

Die hier angegebenen O-Partialantigene ergeben sich aus verschiedenen Absorptions-Versuchen, deren Resultate in der Tabelle 2 angegeben sind

Es geht aus der Tabelle 2 hervor, dass die Bestimmung der O-Untergruppen mit Hilfe von 3 Faktor-Seren 48_2, 48_3 und 48_4 vorgenommen werden kann, und zwar mit Hilfe der Objektglas-Agglutination

Kultur	48	48_3	48_4
S dahlem	++	—	—
S djakarta	++	++	—
Arizona 48	++	++	—
Citrobacter 2624/36	—	++	++

Zur Diagnose der verschiedenen *Salmonella-species* der O Gruppe 48 wird empfohlen, ein polyvalentes O Serum von *S dahlem* + *S djakarta* oder Arizona 48 zu benutzen

Lässt man die *Citrobacter* Kulturen ausser Betracht, so kann man die beiden Untergruppen vereinfacht mit 48_1 = *S dahlem* und 48_1, 48_3 = *S djakarta* angeben, gebraucht also zur Diagnose nur ein einziges Faktor-Serum 48_3 (*S djakarta* O-Serum absorbiert mit *S dahlem*)

Die O Gruppe 50

Zu dieser O Gruppe gehoren bisher folgende O Untergruppen
50_1 50_2, 50_4 = *S greenside* und *S Krugersdorp*
50_1, 50_2, 50_3 = *S bonaire* und *S wassenaar*
50_1, 50_3 = *S hooggraven*
Ferner gehoren hierher die Arizona *species*
9a 9b = 50_1, 50_2, 50_3 (= 50 G –)
9a, 9c = 50_1, 50_2 50_4 (= 50 1 z)

Die O Identität von *S arizonae* 9a 9c mit *S greenside* war bereits in der Arbeit von *Schrire, Kauffmann & Edwards* mitgeteilt worden

TABELLE 3
O Gruppe 50

Kultur	O-Seren				
	S greenside			S hooggraven	
	Nicht absorb	Absorbiert mit		Nicht absorb	Absorb mit S greenside
		S hooggraven	E 55 B5		
S greenside	1280	512	128	160	0
S hooggraven	80	0	0	1280	512
Ar 9a 9b	1280	512	0	1280	512
Ar 9a 9c	320	256	16	160	0
E coli 55 B5	2560	1024	0	320	0
Faktoren	50_1 50_2 50_4	50 50_4	(50_4)	50_1 50_3	50_3

Das O Serum von S *hooggraven* wurde durch Arizona 9a 9b völlig absorbiert

Kultur	O-Seren				
	Arizona 9a 9b			Arizona 9a 9c	
	Nicht absorb	Absorbiert mit		Nicht absorb	Absorb mit Ar 9a 9b
		Ar 9a 9c	S hooggraven		
S greenside	320	0	128	320	64
S hooggraven	320	128	0	320	0
Ar 9a 9b	640	128	256	640	0
Ar 9a 9c	160	0	32	320	64
E coli 55 B5	640	0	256	1280	128
Faktoren	50_1 50 50_3	50_3	50_2	50_1 50_2 50_4	50_4

Das O S [illegible]

da [illegible]
m [illegible]
Al [illegible]

Die O-Identität von „Arizona" 9a, 9b mit *S wassenaar* wurde erst jetzt von uns festgestellt Es besteht jedoch nicht nur O-Identität zwischen diesen beiden *species*, sondern diese *species* enthalten auch dieselben H-Antigene und gehören zu demselben Biotyp Es handelt sich also um ein und dieselbe *species*, die zum *sub-genus* II gerechnet wird Es sei aber betont, dass es sich hierbei um einen stark abweichenden Biotyp des *sub-genus* II handelt, da die Reaktionen in Dulcit, Mukat und Malonat negativ sind, während der KCN-Test positiv ist und Salicin nach 1–2 Tagen gespalten wird

Ferner gehört zur O-Gruppe 50 – wie seit langem bekannt – die *Escherichia-species* 55. B5 6, deren O-Antigene mit 50_1, 50_2, (50_4) angegeben werden können

Zwecks Vereinfachung kann man den 50_4-Faktor auslassen, sodass die Antigenstruktur wie folgt angegeben werden kann

50_1, 50_2 = *S greenside*, *S krugersdorp*, Arizona 9a, 9c und *Escherichia* 55. B5

50_1, 50_2, 50_3 = *S bonaire* und *S wassenaar*

50_1, 50_3 = *S hooggraven*

Die „Arizona"-Kultur 9a, 9b wurde aus dem obigen Schema ausgelassen, da sie mit *S wassenaar* identisch ist Zur Differentialdiagnose dieser 3 O-Untergruppen benötigt man also nur 2 Faktor-Seren 50_2 und 50_3

Diese Befunde stimmen mit den Angaben von *Guinee, Kampelmacher & Willems*, die *S wassenaar* und *S bonaire* beschrieben, überein Die Autoren fanden, dass *S wassenaar* und *S bonaire* sich gleich verhielten und von *S greenside* verschieden waren Es wurden aber keine Versuche, aus denen die O-Identität von *S wassenaar* und *S bonaire* hervorging, mitgeteilt

Die hier angeführten O-Partialantigene der O-Gruppe 50 ergeben sich aus zahlreichen Absorptions-Versuchen, die zum Teil in Tabelle 3 wiedergegeben sind

Es geht aus der Tabelle 3 hervor, dass die Bestimmung der O-Untergruppen mit Hilfe von 3 Faktor-Seren 50_2, 50_3 und 50_4 vorgenommen werden kann, und zwar mit Hilfe der Objektglas-Agglutination

Kultur	50_2	50_3	50_4
S greenside	++	—	++
S hooggraven	—	++	—
S wassenaar	++	++	—

Faktor-Serum 50_2 = *S wassenaar* O-Serum absorbiert mit *S hooggraven*

Faktor-Serum 50_3 = *S hooggraven* O-Serum absorbiert mit *S greenside*

Faktor-Serum 50_4 = *S greenside* O Serum absorbiert mit *S wassenaar* oder Arizona 9a, 9c O-Serum absorbiert mit Arizona 9a, 9b

Wie es aus Tabelle 3 hervorgeht, sind *S greenside* und *E coli* 55 B5 nicht vollig identisch, da das O-Serum von *E coli* 55 B5 zwar durch *S greenside* vollig absorbiert wird, aber umgekehrt das O-Serum von *S greenside* nicht durch *E coli* 55 B5 erschöpft wird Um diese Differenz zum Ausdruck zu bringen, wurde die Formel von *E coli* 55. B5 mit 50_1, 50_2, (50_4) angegeben

Will man – wie bereits erwähnt – die Diagnose der 3 O-Untergruppen vereinfachen, so kann man den 50_4-Faktor ausser Betracht lassen und die Differentialdiagnose nur mit Hilfe der 50_2 und 50_3 Faktor Seren stellen

S greenside + *S krugersdorp* = 50_1, 50_2
S wassenaar + *S bonaire* = 50_1, 50_2, 50_3
S hooggraven = 50_1, 50_3

Zur Diagnose der verschiedenen *Salmonella species* der O Gruppe 50 wird empfohlen, ein polyvalentes O-Serum von *S greenside* + *S wassenaar* zu benutzen, damit alle 4 Partialfaktoren 50_1, 50_2, 50_3 und 50_4 in diesem Serum vertreten sind

Betreffs der Antigenbeziehungen zwischen *Salmonella* O 50 und Arizona O 9 sei betont, dass die O-Antigene von *S greenside* = 50_1, 50_2, 50_4 mit *S arizonae* 9a, 9c und dass *S wassenaar* = 50_1, 50_2, 50_3 mit „Arizona" 9a, 9b identisch sind Der in der Arbeit von *Westphal, Kauffmann, Lüderitz & Stierlin* untersuchte Arizona Stamm N 99 der O Gruppe 9 hat die Antigenstruktur 9a, 9b und ist deshalb nicht mit *S greenside* identisch Dementsprechend ergab auch die qualitative Bausteinanalyse der Polysaccharide Unterschiede zwischen *S greenside* und Arizona N 99 (= 09) Es sei bei dieser Gelegenheit noch darauf hingewiesen, dass bei einer wiederholten Untersuchung des Arizona N 99-Stammes Fucose nicht nachgewiesen werden konnte (siehe bei *Kauffmann, Jann Kruger, Lüderitz & Westphal*) Der einzige Unterschied zwischen *S greenside* und Arizona N 99 besteht also in dem Fehlen von [illegible] und „Arizona" 9a, 9b andererseits zu denselben Chemo Typen gehoren werden

ZUSAMMENFASSUNG

Auf Grund einer näheren Untersuchung aller bisher bekannt[illegible] ... Seren in der Routine-Diagnose berichtet Im *Kauffmann-White-Schema* bleiben die O-Untergruppen unberucksichtigt sie sollen nur in besonderen, epidemiologisch wichtigen Fallen diagnostiziert werden

Die O-Identität von „Arizona" 9a, 9b mit *S wassenaar* wurde erst jetzt von uns festgestellt. Es besteht jedoch nicht nur O-Identität zwischen diesen beiden *species*, sondern diese *species* enthalten auch dieselben H-Antigene und gehören zu demselben Biotyp. Es handelt sich also um ein und dieselbe *species*, die zum *sub-genus* II gerechnet wird. Es sei aber betont, dass es sich hierbei um einen stark abweichenden Biotyp des *sub-genus* II handelt, da die Reaktionen in Dulcit, Mukat und Malonat negativ sind, während der KCN-Test positiv ist und Salicin nach 1–2 Tagen gespalten wird.

Ferner gehört zur O-Gruppe 50 – wie seit langem bekannt – die *Escherichia-species* 55 : B5 : 6, deren O-Antigene mit 50_1, 50_2, (50_4) angegeben werden können.

Zwecks Vereinfachung kann man den 50_1-Faktor auslassen, sodass die Antigenstruktur wie folgt angegeben werden kann:

50_1, 50_2 = *S greenside*, *S krugersdorp*, Arizona 9a, 9c und *Escherichia* 55 : B5

50_1, 50_2, 50_3 = *S bonaire* und *S wassenaar*

50_1, 50_3 = *S hooggraven*

Die „Arizona"-Kultur 9a, 9b wurde aus dem obigen Schema ausgelassen, da sie mit *S wassenaar* identisch ist. Zur Differentialdiagnose dieser 3 O-Untergruppen benötigt man also nur 2 Faktor-Seren: 50_2 und 50_3.

Diese Befunde stimmen mit den Angaben von *Guinee, Kampelmacher & Willems*, die *S wassenaar* und *S bonaire* beschrieben, überein. Die Autoren fanden, dass *S wassenaar* und *S bonaire* sich gleich verhielten und von *S greenside* verschieden waren. Es wurden aber keine Versuche, aus denen die O-Identität von *S wassenaar* und *S bonaire* hervorging, mitgeteilt.

Die hier angeführten O-Partialantigene der O-Gruppe 50 ergeben sich aus zahlreichen Absorptions-Versuchen, die zum Teil in Tabelle 3 wiedergegeben sind.

Es geht aus der Tabelle 3 hervor, dass die *Bestimmung der O-Untergruppen mit Hilfe von 3 Faktor-Seren 50_2, 50_3 und 50_4 vorgenommen* werden kann, und zwar mit Hilfe der Objektglas-Agglutination.

Kultur	50_2	50_3	50_4
S *greenside*	++	—	++
S *hooggraven*		++	—
S *wassenaar*	++	++	—

Faktor-Serum 50_2 = *S wassenaar* O-Serum absorbiert mit *S hooggraven*

Faktor-Serum 50_3 = *S hooggraven* O-Serum absorbiert mit *S greenside*

Faktor-Serum 50_4 = *S greenside* O-Serum absorbiert mit *S wassenaar* oder Arizona 9a, 9c O-Serum absorbiert mit *Arizona* 9a, 9b

AUS STATENS SERUMINSTITUT
(INTERNATIONALE SALMONELLA CENTRALE), KOPENHAGEN

ZUR DIFFERENTIALDIAGNOSE DER SALMONELLA-SUB GENERA I, II UND III

Von

F. KAUFFMANN

Eingegangen 6. xii. 62

In einer früheren Mitteilung „*Two biochemical sub divisions of the genus Salmonella*" hat der Verfasser das *genus Salmonella* in 2 biochemisch definierte *sub genera* eingeteilt *sub genus* I (typische *Salmonella* Bakterien) und *sub genus* II (atypische *Salmonella* Bakterien) Gleichzeitig wies er darauf hin, dass die Arizona Gruppe als *Salmonella sub genus* III bezeichnet werden konne

Die Differentialdiagnose wurde mit Hilfe von Dulcit, des Gelatine-Testes und der organischen Säuren gestellt Wenn wir nur das typische Verhalten der einzelnen *species* innerhalb der 3 *sub genera* berücksichtigen, so konnen wir ein Schema, das in der folgenden Tabelle wiedergegeben ist aufstellen

TABELLE

Typisches Verhalten der Sub Genera

	I	II	III
Dulcit	+1	+1	—
Lactose	—	—	+1 oder x
β Galaktosidase		oder x	+
Gelatine		+	+
d Tartrat	+1	— oder x	oder x
l Weinsäure	d	—	—
i Weinsäure	d	—	—
Natrium Citrat	+1	+2	+2 4
Mukat	+1	+1 ?	d
Malonat		+1 2	+1 2

Zeichenerklärung — = negativ (bei Dulcit und Lactose nach 30 Tagen) + = positiv +1 = positiv nach 1 Tage x = spat und unregelmässig positiv bei β Galaktosidase nach 1 4 Tagen positiv d = differente biochemische Typen

In die Tabelle wurde der β Galaktosidase-Test eingefugt, da auch mit seiner Hilfe Unterschiede zwischen den 3 *sub-genera* nachweisbar sind Dieser Test wurde kurzlich von *Le Minor & Ben Ham*

LITERATUR

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Kauffmann F & *Vandepitte J* A new Salmonella Type (43 y 1 5) from the Belgian Congo Acta path et microbiol scandinav *35* 71 1954
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ZUR DIFFERENTIALDIAGNOSE DER SALMONELLA-SUB GENERA I, II UND III

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Die Differentialdiagnose wurde mit Hilfe von Dulcit, des Gelatine Testes und der organischen Säuren gestellt Wenn wir nur das typische Verhalten der einzelnen *species* innerhalb der 3 *sub genera* berücksichtigen so können wir ein Schema, das in der folgenden Tabelle wiedergegeben ist, aufstellen

TABELLE
Typisches Verhalten der Sub Genera

	I	II	III
Dulcit	$+^{1}$	$+^{1}$	—
Lactose	—	—	$+^{1}$ oder x
β Galaktosidase	—	oder x	+
Gelatine		+	+
d Tartrat	$+^{1}$	— oder x	— oder x
l Weinsäure	d	—	—
i Weinsäure	d	—	—
Natrium-Citrat	$+^{1}$	$+^{2}$	$+^{2\ 4}$
Mukat	$+^{1}$	$+^{1\ 7}$	d
Malonat	—	$+^{1\ 2}$	$+^{1-2}$

Zeichenerklärung — = negativ (bei Dulcit und Lactose nach 30 Tagen), + = positiv $+^{1}$ = positiv nach 1 Tage x = spät und unregelmässig positiv, bei β Galaktosidase nach 1 4 Tagen positiv d = differente biochemische Typen

In die Tabelle wurde der β Galaktosidase Test eingefügt, da auch mit seiner Hilfe Unterschiede zwischen den 3 *sub-genera* nachweisbar sind Dieser Test wurde kürzlich von Le Minor

LITERATUR

Erber M & Kauffmann F A new Salmonella type (Salmonella djakarta) = 31 z_4 z_{24} Acta path et microbiol scandinav *41* 79 80 1957

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Westphal O Kauffmann F Lüderitz O & Stierlin H Zur Immunchemie der O Antigene von Enterobacteriaceae III Analyse der Zuckerbausteine kreuzreagierender Salmonella Arizona und Escherichia O Antigene" Zbl Bakt I Orig *179* 336 342 1960

Bei Ablesung nach 1 Tage ergaben also 41 Kulturen von 59 positiven Kulturen eine positive Reaktion. Um hiervon einige Beispiele zu geben, seien folgende Resultate angeführt:

Sub Genus II	Nach 3 Stunden	1 Tag	4 Tagen
S. detroit	—	—	—
S. humber	—	—	—
S. uphill	—	—	—
S. stellenbosch	—	—	+
S. westpark	—	—	+
S. bulawayo	±	+	+
S. ch. novum	±	+	+
S. tranoroa	±	+	+

Von den 51 Kulturen des *sub genus* III ergaben alle eine positive Reaktion und zwar bereits nach 1–3 Stunden. Nur 4 Kulturen ergaben nach 3 Stunden eine ± Reaktion, waren aber alle nach 1 Tage +. Die 6 biochemisch abweichenden Kulturen, die zum *sub genus* II gerechnet wurden, reagierten alle negativ nach 4 Tagen.

Während diese 6 Kulturen früher zur Arizona Gruppe gerechnet wurden, fasst der Verfasser sie als atypische *sub genus* II *species* auf. Die biochemischen Abweichungen dieser Kulturen sind in folgendem Schema wiedergegeben:

	Salicin	Mukat	Malonat	KCN	Sorbit
1	$+^{1}$	—	—	+	+
2	$+^{2}$	—	—	+	+
3	$+^{1}$	—	—	+	+
4	+	—	—	+	+
5	$+^{2}$	—	—	—	+
6	—	$+^{3}$	—	—	—

Alle 6 Kulturen waren in Dulcit und Lactose sowie im β Galaktosidase Test negativ und verflüssigten Gelatine. d Tartrat = — oder x, l und i Weinsäure = negativ, Natrium Citrat = $+^{2}$, doch bei Nr. 5 negativ.

Das serologische Verhalten dieser Kulturen ist in folgender Zusammenstellung gegeben:

1 — Arizona 396–56 = 10a, 10c, 13, 15 = Sal. 40, G
2 Arizona 1450, 53 = 21, 1, 2, 6 = Sal. 43, z_4
3 Arizona 2959, 55 = 22, 1, 2, 5, 6 = Sal. 21, z_4
4 — Arizona N 99 = 9a, 9b, 13, 15 — = Sal. 50, G —
5 Arizona 1675–55 = 32, 1, 2, 6 — = Sal. C, z_4 —
6 — Arizona 142, 56 = 11, 3, 28 = Sal. 45, a, e, n

(Näheres siehe bei *F. Kauffmann & R. Rohde*)

O N P G	80 mg
aq dest	15 ml
Pufferlösung	5 ml

O N P G = Orthonitrophenyl β D Galaktopyranosid von Light & Co Colnbrook Bucks, England

Pufferlösung	
$NaH_2PO_4H_2S$	6 9 gr
aq dest	40 ml
5n NaOH	7 5 ml
pH 7 0	
aq dest ad	50 ml

Die zu untersuchenden Kulturen wurden auf 1 % Lactoseagar-Platten 24 Std bei 37° C gezüchtet und pro Petrischale in 1 ml NaCl Lösung aufgeschwemmt Es wurden dann 0,2 ml Toluol hinzugefügt und ½ Std bei 37° C im Thermostat bebrütet Dann wurde zu jedem Glase 1 ml der obigen O N P G -Lösung gegeben, worauf die Röhrchen wieder in den Thermostaten von 37° C kamen Sie wurden nach 1, 3 und 24 Stunden sowie nach 2, 3 und 4 Tagen abgelesen Eine starke Gelbfärbung wurde mit +, eine schwache mit ± bezeichnet

Im ganzen wurden 334 Kulturen untersucht

140 Kulturen des *sub-genus* I, und zwar 111 Kulturen, die Gelatine nicht verflüssigten und 29 Kulturen, die Gelatine verflüssigten, also atypisch waren Es wurden Vertreter aller O Gruppen ausgewählt, sofern solche des *sub-genus* I vorhanden waren Bei den häufigen O Gruppen B, C, D und E, den klassichen *Salmonella* O-Gruppen, wurden zahlreiche *species* untersucht

143 Kulturen des *sub-genus* II, und zwar alle bis Ende 1962 bekannten *species*, meist nur 1 Vertreter einer *species* Hierzu gehören 6 biochemisch atypische Kulturen, die früher zur Arizona-Gruppe gerechnet wurden (siehe weiter unten)

51 Kulturen des *sub-genus* III, die biochemisch typische Arizona Kulturen waren und Lactose meist prompt oder verzögert spalteten

Von den 140 Kulturen des *sub-genus* I ergaben 137 Kulturen eine negative Reaktion im β-Galaktosidase-Test nach 4 Tagen *S jukestown* = 13,23 i e,n,z15, die Gelatine nicht verflüssigte, gab nach 2–4 Tagen eine positive Reaktion, also stark verzögert Von den 29 Kulturen des *sub genus* I, welche Gelatine verflüssigten, ergaben 2 *species* eine positive Reaktion im β-Galaktosidase-Test *S wayne* war nach 24 Stunden positiv, während *S ferlac*, die prompt Lactose spaltete, nach 1 Stunde positiv war

Von den 143 Kulturen des *sub-genus* II ergaben 84 Kulturen eine negative Reaktion, während 59 Kulturen positiv reagierten, und zwar

4 Kulturen nach 1–3 Stunden,
37 Kulturen nach 1 Tage und
18 Kulturen nach 2–4 Tagen

genus Salmonella nur biochemisch definieren will, so kommen die 3 *sub genera* als *species* in Betracht Der Verfasser hält aber dieses nicht für zweckmässig und hat im Sinne der modernen Klassifikation die *species* als eine Gruppe verwandter, sero-fermentativer Phag-Typen definiert

ZUSAMMENFASSUNG

Es wird über die Differentialdiagnose zwischen den *sub-genera* I, II und III des *genus Salmonella* berichtet, speziell über den β-Galaktosidase Test Dieser gab beim *sub-genus* I negative, beim *sub genus* II teils negative, teils verzögert positive und beim *sub-genus* III prompt positive Resultate Das *sub-genus* II steht also auch in dieser Hinsicht zwischen den *sub-genera* I und III, von denen es aber deutlich verschieden ist

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Nachdem bereits Nr 4 = Arizona N 99 = Sal 50 G – auf Grund ihrer Identität mit *S wassenaar* aus der Arizona-Gruppe ausgeschlossen und in das *sub-genus* II überführt wurde, sollen jetzt auch die übrigen 5 *species* als *sub-genus* II-Kulturen betrachtet werden

Auf Grund ihres negativen Verhaltens in Malonat stehen diese atypischen *species* zwischen den *sub-genera* I und II, sodass sie also nicht zum *sub-genus* III gehören Da wahrscheinlich noch weitere, derartige *species* vorkommen, so wird es bald zweckmässig sein, diese in einem neuen *sub-genus* zu vereinen, doch können sie vorläufig als atypische *sub-genus* II-*species* betrachtet werden Dass es sich hier wirklich um eine zwischen *sub-genus* I und II stehende Gruppe handelt, zeigt das Vorkommen malonat-negativer und gelatine-verflüssigender *species* innerhalb des *sub-genus* I, z B *S schleissheim, S abortus bovis* etc

Die Sonderstellung dieser atypischen *sub-genus* II-*species* beruht auf folgenden Merkmalen

Negatives Verhalten in Dulcit, Lactose, Mukat und Malonat sowie im β-Galaktosidase-Test

Positives Verhalten in KCN, Salicin und Gelatine

Das typische Verhalten der 3 *sub-genera* im β-Galaktosidase-Test ist in folgendem Schema wiedergegeben

Nach	*Sub Genera*			
	I	II		III
	ca 100%	ca 60%	ca 40%	ca 100%
1 Stunde	—	—	(±)	+
3 Stunden	—	—	±	+
1 Tage	—	—	+	+
4 Tagen	—	—	+	+

Es geht auch aus diesem Schema hervor, dass das *sub-genus* II eine Zwischenstellung zwischen den *sub-genera I* und *III einnimmt und* vom *sub-genus* III verschieden ist

Vergleicht man die Ergebnisse des β-Galaktosidase-Testes mit denen des üblichen Lactose-Testes, sieht man, dass die Ergebnisse beim *sub genus* I identisch sind Auch beim *sub-genus* III stimmen die Resultate prinzipiell überein, da alle hier untersuchten Arizona-Kulturen Lactose prompt oder verzögert spalteten Mit Hilfe des β Galaktosidase-Testes kann man die positive Reaktion bereits nach wenigen Stunden ablesen Beim *sub-genus* II, das einen negativen Lactose-Test ergibt, fallen die Resultate mit dem β Galaktosidase-Test verschieden aus, da ca 60 % der Kulturen negativ und ca 40 % verzögert positiv reagierten

Zur Diagnose der 3 *sub-genera* ist aber dieser Test entbehrlich, da wir mit Hilfe vom Lactose, Dulcit, Gelatine, d-Tartrat und Malonat die Diagnose stellen können

Falls man im Sinne der orthodoxen Klassifikation die *species* des

TABLE 1

The Original Specimens from which the Staphylococci Were Isolated

Origin	No of GV strains	No of "normal" strains
Pus from wounds	11	7
Nose and throat swabs	6	10
Ear discharge	3	4
Sputum	1	2
Pleural fluid	1	1
Blood		1
Pus from conjunctiva		1
Prostatic discharge		1
Blood at autopsy	1	
Spleen at autopsy	1	

METHODS

The coagulase reaction was performed as a slide test

Pigment production was examined by inoculation on Löffler's serum agar The tubes were kept at room temperature for six days and subsequently read

Fermentation of sucrose, mannitol and lactose was studied by inoculation with 0.02 ml of an 18 hour broth culture in each tube Readings were carried out after 24 48 and 72 hours

Sensitivity to antibiotics was determined by the paper disk method (6) The strains considered resistant were those which were not inhibited by the following concentrations penicillin 2 units per ml, erythromycin 4 µg per ml streptomycin 32 µg per ml the tetracycline group 4 µg per ml chloramphenicol 32 µg per ml, novobiocin 16 µg per ml, and sulphonamides 6 mg per 100 ml

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...of disk with penicillin was prepared for each plate The plates were kept at room temperature for three hours to allow penicillin diffusion after which they were incubated overnight at 37° C

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th... ...es according to ...man & Williams (5) Strains giving no lysis with the routine test dilution (RTD) of the phages were also tested at 1000 × RTD When no lysis was obtained with 1000 × RTD the strain was considered nontypable (NT)

FROM THE DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF UPPSALA AND THE HOSPITAL FOR INFECTIOUS DISEASES, UPPSALA, SWEDEN

STRAINS OF *STAPHYLOCOCCUS AUREUS* WITH INCREASED TOLERANCE TO GENTIAN VIOLET

By

FOLKE NORDBRING

Received 20.viii.62

The inhibiting effect of gentian violet, *even in low* concentrations on the growth of *Staphylococcus aureus* has been used in routine bacteriologic work in many laboratories. By adding gentian violet to the blood agar plates, most strains of *Staph. aureus* are completely inhibited. In the laboratory of the Uppsala Department of Bacteriology, gentian violet is added to the sheep blood agar medium in a concentration of approximately 1.3 μg of gentian violet per ml of medium.

In this laboratory on the contrary, it has been observed occasionally in recent years that some strains of *Staph. aureus* grow abundantly on the blood agar plates with gentian violet added. These colonies are round and opaque, they are smaller than the normal colonies on blood agar plates and have a faint blue colour. If the strain is haemolytic, a distinct zone of haemolysis surrounds the colony and the staphylococci might well be mistaken for haemolytic streptococci.

Such strains of *Staph. aureus* growing on gentian violet plates have been investigated with regard to their morphologic and other characteristics, resistance to antibiotics and gentian violet, penicillinase production and bacteriophage types. The results are reported here.

MATERIAL

Twenty four strains of *Staph. aureus* growing on gentian violet plates (GV strains) were studied. The original specimens sent to the laboratory for examination are listed in Table 1. These strains were derived from 19 patients from several hospitals in different cities. All were resistant to penicillin (benzyl penicillin) according to the paper disk diffusion method of *Ericsson, Högman & Wickman* (6).

Twenty seven strains of *Staph. aureus* which failed to grow on gentian violet plates ("normal" strains) were investigated at the same time. These strains originated from various sources (listed in Table 1) and were chosen at random among the routine laboratory specimens during the same period as the GV strains (Oct.–Nov. 1959). Twenty five of the "normal" strains were resistant to penicillin according to the paper disk method, one strain being isolated from a person who also had a GV strain. Two "normal" strains were sensitive to penicillin, one being isolated form a person with a simultaneous GV strain. The Oxford strain 209 was used as a control.

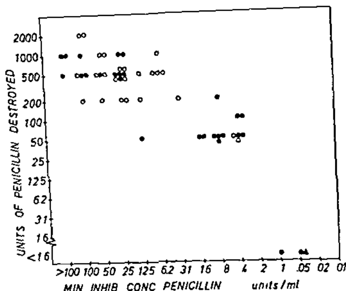


Fig 2

The amount of penicillin destroyed by 0 5 ml of broth culture Symbols as in Fig 1

tance is more pronounced among the GV strains The minimal inhibitory concentration of penicillin is 3 1 units per ml or higher for 14 (36 per cent) of the "normal" strains and for 22 (92 per cent) of the GV strains

It is seen from the figure that all of the "normal" strains are inhibited by 0 2 0 4 μg of gentian violet per ml except one strain which tolerates 0 8 μg per ml The GV strains tolerate a considerably higher concentration of gentian violet Most GV strains are inhibited by 1 6–3 1 μg per ml (one strain tolerates 6 2 μg per ml) *i e* the "normal" strains are 4 15 times more sensitive to gentian violet than the GV strains

The amount of penicillin destroyed by 0 5 ml of broth culture of the different strains (approximately 500 million organisms) in one hour of incubation which indirectly represents the penicillinase production is shown in Fig 2 It is readily seen from the figure that there is a fairly good correlation between the extent of penicillin resistance and penicillinase production and there is no difference between GV strains and "normal" strains No measurable amount of penicillin is destroyed by the three strains susceptible to penicillin Most strains inhibited by 0 4 1 6 units per ml destroy 50 100 units and most strains requiring 3 1 units per ml or more for inhibition destroy 200 2000 units

The obvious tendency to pronounced resistance to penicillin among the GV strains raised the question whether these strains were also particularly prone to resist other antibiotics Table 2 gives the results of the sensitivity tests according to the paper disk method It is apparent

RESULTS

All of the strains produced coagulase Two of the GV strains and two of the "normal" strains were non-haemolytic There was no difference between the two types of staphylococci as regards the appearance of colonies on blood agar and the pigment production Most strains exhibited a heavy gold pigmentation All of the strains fermented sucrose, mannitol, and lactose without production of gas

The ability of the GV strains to grow on gentian violet agar seemed to be a stable property The GV strains were kept in the refrigerator on blood agar plates for five months with frequent re-inoculations without losing their increased tolerance to gentian violet Four GV strains were inoculated on gentian violet plates with seven passages in five weeks without losing their capacity to grow Thus a continuous contact with gentian violet for several weeks could not provoke a change in tolerance

The results of the tube tests of susceptibility to penicillin and gentian violet are given in Fig 1 The two "normal" strains, sensitive to penicillin according to the paper disk method, and the Oxford strain 209 are inhibited by 0 05–0 1 units of penicillin per ml The remaining strains display a varying degree of resistance to penicillin under the standardized experimental conditions, although the tendency to resis

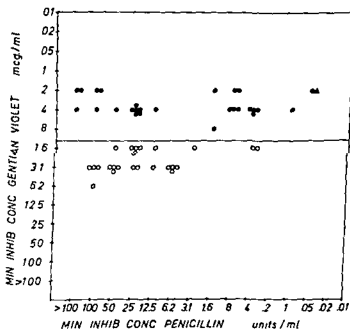


Fig 1

Results from the serial dilution tests of the sensibility to penicillin and gentian violet ● denotes 'normal' strains ○ GV strains and ▲ the Oxford strain 209 The horizontal line indicates the concentration of gentian violet in the blood agar plates with gentian violet added to the medium

TABLE 3

Bacteriophage Patterns of the Strains Studied

No of 1000x RTD	Phage pattern	Phage group	No of GV strains	No of normal strains
	Nt			1
	187			1
	52/80	I		2
	52/80/81	I		4
	80/81/KS6	I		5
1	34/3C	II		3
	3B/3C/55/71	II		1
	53	III	1	
7	54	III	7	
2	6/54	III	2	
	53/54	III	1	
1	54/819	III	1	
2	54/1034	III	2	
	6 47/53	III		1
1	6/53/54	III	1	
1	7/47/54	III	1	
	54/75/77	III		1
1	6 53 54/1034	III	1	
	7/42E/54/819	III		1
1	6/7/47/53/54	III		1
1	6/7/54 819/1034	III	1	
1	6/53 54/77/819	III		1
1	7/42E/54/819/1034	III	1	
1	7/42E/47/54 819 1034	III	1	
1	6 7/53/54/819/1034	III	1	
4	Other patterns	III		5
1	29/42E,53/54	I+III	1	
2	29 42E/53/54/1034	I+III	2	

It is known from the studies by *Park & Strominger* (12, 13, 14) that the inhibiting effect on staphylococci of gentian violet is brought about by its interfering at some point in the reaction sequence leading to cell wall synthesis This action is similar to that of penicillin, but gentian violet appears to interfere at an earlier stage in the biosynthetic sequence The mechanism behind the manifold increase in tolerance to gentian violet of the GV strains remains to be elucidated The resistance to penicillin of the GV strains as expected, was caused by penicillin destruction by penicillinase Further studies are needed to determine whether GV strains occur exclusively among staphylococci of phage group III

The capacity to tolerate larger concentrations of gentian violet than normal' strains seemed to be a stable property The capacity did not vanish after several transfers in the laboratory In two cases the same GV strains were isolated from the same lesion on separate occasions

from the table that the GV strains have a marked tendency to resistance to various antibiotics and the percentage of resistant GV strains is higher than that of "normal" strains, with the exception of penicillin and the sulphonamides The resistance of the GV strains to the tetracyclines is particularly marked It is also notable that 50 per cent of the GV strains are resistant to erythromycin, while only 12 per cent (three strains) of the "normal" ones are resistant to this drug

The phage patterns of the strains are recorded in Table 3 It is seen from the table that the "normal" strains are scattered throughout the different phage groups, while all of the GV strains fall into phage group III with various patterns It is noteworthy that a high proportion of the GV strains (22 out of 24) gave lysis only with 1000 × RTD

TABLE 2

Results from the Susceptibility Tests According to the Paper Disk Method Two Normal Strains Sensitive to Penicillin Are Excluded

Agent	Resistant to			
	GV strains		Normal strains	
	No	%	No	%
Sulphonamides	21	87	22	88
Penicillin	24	100	25	100
Erythromycin	12	50	3	12
Streptomycin	17	71	8	32
Chlortetracycline	20	83	10	40
Oxytetracycline	21	87	10	40
Tetracycline	20	83	7	28
Chloramphenicol	9	37	3	12
Novobiocin	9	37	3	12

DISCUSSION

It has been observed by many workers that staphylococci resistant to several antibiotics are almost completely confined to phage group III (1, 3, 4, 9, 11, 15) It has also been particularly pointed out in some studies that the majority of erythromycin resistant staphylococci fall within group III (7, 8, 10) *Barber & Whitehead* (2) suggested in 1949 that group III staphylococci are genetically less stable than other strains and that resistant mutants develop more readily

The strains of staphylococci with increased tolerance to gentian violet investigated in the present study were all found to react with bacteriophages of group III and they were all resistant to at least two antibiotics out of the eight tested Nineteen out of 24 strains (80 per cent) were resistant to five or more antibiotics This suggests that development of multiple antibiotic resistance may be accompanied by increased resistance to gentian violet

relapse, the patient had staphylococcal skin boils Naturally, the bone fistula might have been infected via the skin The rapid disclosure that the fistula strain grew on gentian violet agar, while the strain from the skin abscesses did not, revealed that the two sites of infection were not epidemiologically connected with each other This could be stated after an overnight incubation, long before the phage-typing was performed

In fact, the property of tolerance to increased concentration of gentian violet could be used as a means by which to mark staphylococci, together with phage-typing, in epidemiologic studies of various kinds

SUMMARY

Strains of staphylococci which grow abundantly on blood agar with gentian violet added (GV strains) have been studied These strains may be mistaken for beta haemolytic streptococci They were found to tolerate 4–15 times higher concentrations of gentian violet than other "normal" strains of staphylococci chosen at random among the routine laboratory specimens All GV strains studied reacted with bacteriophages of group III and exhibited a marked tendency to multiple resistance to antibiotics

The GV strains retained their capacity to grow on gentian violet agar after several transfers in the laboratory A case report is given, demonstrating a long standing infection with a GV strain It is pointed out that recognition of GV strains may be of value in epidemiologic work

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10 Maccabe ... cus ...

The following case history gives a good illustration of a GV strain, which persisted in the tissues for years

Case History

F N, a woman 61 years old In July 1958 supracondylar transverse fracture of the right femur Operative reposition and fixation of fragments by metal plate and wire was performed There was a slow but uneventful healing and the fracture seemed to be consolidated one year later In Sept 1959 signs of infection were noted and a large abscess cavity around the osteosynthesis area was emptied The osteosynthesis material was removed A continuously suppurating fistula developed and during the following year the patient was given various antibiotics and sequesters were repeatedly removed *Staph aureus* was isolated many times from the fistula (phage pattern 1000 × RTD 6/7/47/53/54/75/77, group III) and from the nose and throat (phage type KS6/80/81, group I)

In Oct 1960 a first course of methicillin (Belfacillin® Astra) was given The drug was administered for two weeks and cultures from the fistula secretion were negative after 12 days remaining so for seven weeks although there was still discharge from the fistula *Staph aureus* was isolated from the fistula in Dec 1960 and again the phage pattern was 1000 × RTD 6/7/47/53/54/75/77 At the same time the patient developed skin abscesses with growth of *Staph aureus* but of another phage pattern KS6/80/81

A second course of methicillin was started in Dec 1960 lasting for seven weeks The patient was again free from all staphylococci after 12 days and remained so for 10 weeks There was still a moderate secretion from the fistula Staphylococci of the same phage pattern as previously seen (1000 × RTD 6/47/53/54/75/77) were again isolated from the fistula secretion in March 1961

A third course of methicillin began in March 1961 and continued for eight weeks The bacterial cultures from the fistula discharge were negative on the eighth day and the patient was free from staphylococci for 10 weeks Surgical revision of the osteitis focus was carried out In June 1961 the same type of *Staph aureus* reappeared in the pus (1000 × RTD 6/7/47/53/54/75/77) A skin furuncle developed some days later but from this focus of infection as before another type of *Staph aureus* was isolated (KS6/80 81)

Following two weeks of constant growth of staphylococci of the same type in the fistula secretion a fourth course of methicillin was given for eight weeks from June 1961 The result was as the previous one The staphylococci disappeared as from the twelfth day but this time the pus remained sterile for six and a half months During the last two months of this period the patient suffered from several skin boils due to *Staph aureus* 80/81 and this type was frequently cultured from the nose and throat but the fistula secretion remained sterile However in Jan 1962 the now familiar type of *Staph aureus* (1000 × RTD 6/47/53/54/75/77) was isolated from the fistula

A fifth course of methicillin followed by oral administration of methyl phenyl isoxazolyl penicillin (Micropenin® Kabi) was started in Febr 1962 with the same quick response as before the cultures being sterile as from the twelfth day

The staphylococci of group III in the above case, isolated from the bone fistula, grew well on gentian violet plates in Dec 1960 and in Febr 1962 and it is reasonable to believe that the same result would have been obtained earlier, had the strains been investigated in this respect The staphylococci in this case had obviously been harboured in the bone for years were capable of re-growth some time after penicillin treatment, and, had retained their property of increased tolerance to gentian violet

The above case history also illustrates the fact that the recognition of GV strains may be useful epidemiologically In connection with some of the relapses of the bone infection, or immediately preceding such

FROM THE INSTITUTE OF CLINICAL BACTERIOLOGY UNIVERSITY OF LUND GENERAL HOSPITAL MALMÖ SWEDEN

STUDIES OF ANTIBODIES IN HUMAN LISTERIOSIS

I *Antibodies in Cases of Bacteriologically Verified Human Listeriosis*

By

STEN WINBLAD

Received 8 xii 62

The discovery that Listeria monocytogenes (L m) may cause septic disease in animals was made by *Gustav Hulphers* (8) who in 1911 described the first case of such illness in animals Listeriosis may affect various animals such as sheep rabbits guineapigs fowls horses cattle chinchillas etc The fact that man too can be thus infected has been known for a long time Usually the manifestations of such an infection are purulent meningitis or general sepsis in newborn infants or in adults meningitis but also anginous septic and oculo glandular forms have been described Human listeriosis attracts special interest when occurring pregnancy or in the neonatal period As a result of *Seeliger's* (19) extensive studies of L m and its infections numerous cases of human listeriosis could be identified in West Germany and recently in other countries too (3 6 7) From Israel has been reported an interesting accumulation of febrile abortions due to infection with L m (*Rappaport et al* 18)

In Denmark *Nyfeldt* (14) claimed to have isolated L m from the blood of patients suffering from infectious mononucleosis during an epidemic in 1929 1930 Nyfeldt regarded L m as the cause of this outbreak but this opinion was disputed by many and no later epidemics of this type have been found In view of our present knowledge however the possibility that Nyfeldt was right after all cannot be entirely discarded

In 1958 the first case in Sweden to be histo pathologically and bacteriologically verified was observed (*Linell Schuller & Winblad* 12) A newborn full term child was taken ill when seven days old and died when nine days old The mother was healthy [illegible] nt studies have [illegible] den during No [illegible] Table 1 where [illegible] cases belonged to the prenatal or neonatal period while 15 cases occurred in adults causing meningitis or pleurisy and 5 other cases are associated with the occurrence of bacteria in the placenta or the meconium of healthy children with healthy mothers (*I au*

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Seeliger & Sulzbacher (21) claim that L. m. on the one hand has antigens in common with Staphylococcus aureus and enterococci on the other. Consequently they consider that an agglutination test for L. m. must be preceded by absorption of the patient's serum with staphylococci or enterococci in order to be of any diagnostic value. The risk for such overlapping reactions, however, is said to be greater in the agglutination than in the complement fixation test. Further proof that the three bacterial groups have antigens in common is offered by *Neter, Anzai & Gorzynski* (13) who found cross-reactions between Staphylococcus aureus and L. m. when studied with a passive haemagglutination technique. *Welshimer* (23) on the other hand, could detect no such common antigens. Experimenting with rabbits he found no L. m. agglutinins in animals immunized against staphylococci and no staphylococcal agglutinins in those rabbits that had been immunized against L. m. At the present moment there are thus divergent opinions concerning the existence of cross reactions between L. m. and other Gram-positive bacteria.

The object of the present investigation is to study the development of antibodies in human infection with L. m. using O-agglutinins and complement-fixing antibodies. The first step here will be to examine the antibodies in bacteriologically verified cases, and the second step to study their presence in a larger group of patients with other clinical conditions as well as in healthy subjects. These matters will obviously have to be elucidated before it can be decided whether the occurrence of antibodies in human serum justifies a conclusive diagnosis of listeriosis in those cases where a culture either is not effected or does not yield positive results. The cultivation of L. m. in cases of clearly established septicaemia or purulent meningitis appears to be fairly simple [illegible]

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METHODS

Antigens for O Agglutination

Listeria m[illegible]

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Dextrose 1[illegible]

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is harvest[illegible] [illegible] [illegible]ional buffer. The s[illegible]

steamed f[illegible] 1 hour and then [illegible]

and stored at +4° C [illegible]

found suitable for ag[illegible]

100 ml of veronal bu[illegible]

which is prevented by the heating [illegible] spontaneous agglutination

---

[1] Kindly supplied by Dr. *Seeliger*, Bonn, for which I wish to express my gratitude.

rell 11) A detailed description of these cases would be beyond the scope of this work, and some of them have been published earlier (1, 2, 4 5, 9, 10, 12 & 22)

As evidenced by *Seeliger's* investigations, L m possesses identifiable antigens of the O and H types Seeliger's use of absorbed immune sera from rabbits led to the recognition of five types of the organism 1, 2 3 4A and 4B

TABLE 1

Cases of Human Listeriosis in Sweden (Nov 1958–April 1962)
Bacteriologically Verified Cases

1 *Foetus or newborn children*

	Number	Maturity	Prematurity	Sick mothers	Healthy mothers	•
Meningitis or Septicemia	12	8	4	5	7	–
Abortion	3		–	1	2	
Stillbirth	6	2	4	3	1	2
Asphyxia *Dead*	2	–	2	2	–	–
Nosocomial infection in maternity hospital	2	2			2	–
	25	12	10	11	12	2

2 *Adults*

	Number	Healthy	Dead
Meningitis	13	9	4
Pleurisy	1	–	1
Septichaemia	1		1
	15	9	6

Total cases 40

3 Bacteria in meconium or placenta Healthy children (*Laurell*)	5

Total bacterial cases 45

Antibodies in man unfortunately provide no conclusive evidence for a satisfactory diagnosis However, *Seeliger* (19) points out that the complement fixation reaction appears to be more specific than a test for agglutinins in serum where even many healthy subjects may show a comparatively high titer (15, 16, 19, & 21) In the opinion of *Poschel & Schwind* (16) a high agglutination titer should be used for diagnostic purposes only in combination with symptoms indicative of listeriosis

Absorptions

Patients sera possessing an agglutination titre of 1/64 or higher have b en absorbed by means of the technique described above In all these cases except one absorption with Staphylococcus aureus and entero cocci was found to lower the agglutination titre against L m by one step only whereas absorption with the Listeria antigen completely re moved the anti Listeria agglutinins In one case an unexpected agglu tination titre against Listeria was found in a child three months old and this titre could be entirely absorbed with enterococci and staphylo-coccus aureus It is obvious from the results obtained however, that there are no significant cross reactions between the Listeria Staphylo-coccus and Enterococcus antigens employed and also that the antigens uesd in this investigation with the methods applied should offer rea sonable protection against the risk of any cross reactions interfering with specificity

The Development of Antibodies in Mothers of Children with Human Listeriosis

In 14 cases of listeriosis in newborn infants and abortions it has been possible to follow the development of antibodies in the mothers These cases showed the following manifestation One of the newborns exhi bited a widespread septicaemia (case 1) In eight cases (cases 2 9) the newborn child had meningitis from which L m could be isolated Two of the cases (10 11) were abortions where L m was isolated from abortion discharges and the vagina In other cases (cases 12 13) a premature child was born who died and from whom L m could be recovered In the last case (case 14) L m was isolated from a stillborn child Thanks to co operative colleagues bloodsamples were obtained from the mothers during the period following parturition making it possible to follow the agglutination in all cases and complement fixa tion titre in 9 cases The maximal titres thus obtained are recorded in Tables 2 and 3

In three cases an agglutination titre of merely 32 was found in three others the titre was 64 while higher agglutination titres were found in the rest Complement fixation tests were performed in 9 cases showing a low titre only once but titres of 1/30 or more in the other nine Comparative studies of antibody titres in healthy subjects (24) prove an agglutination titre of 1 64 or more and a complement fixation titre of 1 30 or more to be titres higher than what is usually found in healthy persons Thus the tabulated titres are obviously elevated more than normally It is also interesting to note that out of those 14 mothers who are included in the investigation only one was treated with chemo therapy immediately after delivery There is thus no reason to suspect a chemotherapeutically induced decrease of antigens The exception is case No 11 in which early antibacterial treatment was given but in

Agglutination Reaction

Inactivated serum is diluted with saline dichotomically 1/8–1/512 each tube containing 0 25 ml 0 25 ml of antigen suspension are added, and the tubes are incubated for 18 hours in a water-bath of 52° C and for another 24 hours in a refrigerator Positive controls with patient serum and rabbit serum are included as well as a negative serum control and a control without serum Reading by means of a handlens, are made, preferably by observing the appearance of the deposit which often gives a sharper end point than reading after shaking the tubes

Absorption Control

Sera with a high agglutination titer are re-examined without absorption after absorption with equal amounts of an autoclaved mixture of Staphylococcus aureus Cowan's types 1, 2, 3 and 4, with a batch of 7 autoclaved enterococcal strains and with the L m antigen respectively.

Antigens for Complement Fixation

The same method is applied as when antigens for agglutination were prepared except that isotonic saline is used for the suspension This preparation is boiled for 30 minutes after which its sterility is tested

Complement Fixation Reaction

Preliminary titrations to determine a suitable dose of the complement fixation antigen are made with a fixed dose of complement and with positive rabbit serum and negative human serum Further tests to ensure reproducibility of results with different antigen batches, are made with positive and negative human sera The other steps of the complement fixation test are carried out as usual

All sera are inactivated at 56° C for 30 minutes

RESULTS

If O antigen was prepared in the manner described above, no spontaneous agglutination could be observed on any occasion

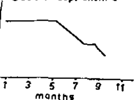
H Agglutinins

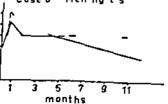
Attempts to establish the presence of agglutinins by means of H antigen according to Seeliger were successful in rabbit immunized against according the different strains, but in human sera the results weredifficult to read and interpret As a consequence, the investigation of antibodies against the H-antigen in human sera was abandoned at an early stage

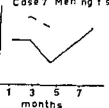
Cross-reactions

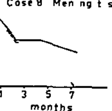
Rabbits which had been immunized against the various types of L m showed good production of O agglutinins and complement-fixing antibodies against L m There was, however, no increase of agglutinins against the mixed antigens of Staphylococcus aureus (O antigens from types 2, 3, and 4) Nor did it exhibit an elevated titre against O antigens from the mixed enterococcal strains *It was also found that rabbits* immunized with O antigens from the above mentioned types of staphylococci completely lacked agglutinins against Listeria Our findings are thus in perfect agreement with *Welshimer*'s (23) in this respect

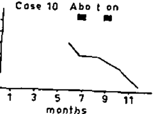
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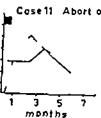




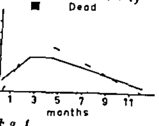


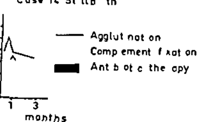












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spite of this no depression of the antibody curve is to be found In three cases, however, antibacterial therapy has been administered in the later stages As seen from Table 3, this investigation shows that an elevated antibody titre, as measured with this technique, is present in agglutination tests in 11 cases out of 14, in complement fixation tests in 8 cases out of 9, and if both antibodies are taken together, in 8 cases out of 9

TABLE 2

Maximal Antibody Titre in Cases with Bacteriologically Verified Human Listeriosis

Case	Disease	Maximal titre in antibody periods		Antibiotic therapy	
		Agglutination	Complement fix	Initially	Late
1	*Mother of newborn with septicaemia*	256		—	
2	Mother of newborn with meningitis	32		—	
3	,	32		—	
4	,	32		—	
5		64	160	—	
6		256	240	—	
7	,	256	240	—	
8	,	512	120	—	
9	,	64	10	—	
10	Mother with abortion	128		—	+
11		128	120	+	
12	Mother of premature child Dead	512	120	—	+
13	,	64	60	—	+
14	,	256	40	—	
15	*Adult Meningitis*	128	30	+	
16		96	240	+	
17		128	0	+	
18		256	20	+	
19		8	40	+	
20	,	32	30	+	

TABLE 3

Frequency of Elevated Titre of Antibodies in Cases of Human Listeriosis

Patients	Number	O-agglutination		Number	Complement fixation		O-agglut C fix
		64	64		30	30	
Mothers of newborn with listeriosis	14	11	3	9	8	1	8
Adults Meningitis	6	4	2	6	4	2	1

Development of Antibodies in Cases of Meningitis in Adults

As many be seen in Table 2, antibody titres here are not increased to the same degree as in mothers of children infected with *Listeria* In 4 cases out of 6, elevated titres are nevertheless present higher than 1/32 in the agglutination test and 1/30 or higher in the complement

likely that in these patients the early antibacterial therapy was responsible for he low titre values In cases 15 and 18, the complement-fixing antibody does assume the form of a curve but the values attained do not amount to the afore-mentioned limit values

The methods employed have thus proved useful in illustrating the development of antibodies in bacteriologically verified cases of listeriosis

DISCUSSION

Purulent meningitis in both adults and newborn children is a serious disease which, in the absence of antibacterial therapy, may lead to death The fact that apparently healthy mothers may give birth to a child or foetus with listeriosis, infected in utero or at parturition, seems to indicate that the mothers have actually been infected themselves although with negligible symptoms There are thus certain similarities between listeriosis and toxoplasmosis in pregnancy In toxoplasmosis serological methods have been of considerable help in the diagnostic procedure and it is therefore to be hoped that such methods in the future will play an equally important rôle in establishing elevated antibody titres against L m

The methods applied in this investigation to determine O agglutinins and complement-fixing antibodies against L m proved elevated antibody titres to develop in most cases provided that no early antibacterial therapy is given that reduces the stimulating effects of antigen on antibody formation It was also found that on the whole these antibody curves agree with those of other specific antibodies in bacterial diseases, except that the elevated titres tend to remain for a long time

By the same procedure, no certain cross-agglutination between L m O antigen on the one hand and Staphylococcus aureus or enterococci on the other could be demonstrated but in spite of this, it is possible that complement fixing antibodies may be more specific than agglutinins, as was proposed by *Seeliger* Further methods for a serological diagnosis of human listeriosis are to be awaited and hoped for

SUMMARY

The development and successive course of O agglutinins and complement-fixing antibodies have been studied in 14 mothers of full term or abortive children with listeriosis, and in 6 adult cases of Listeria meningitis In 11 of the mothers the O agglutination titre rose to 1/64 or more, and in 8 cases out of 9 the complement fixation titre was 1/30 or more The antibody curves in the mothers developed in a manner similar to that of specific antibodies in other bacterial diseases but were characterized by a slow retardation In these cases antibacterial therapy had not been administered during the acute stage Six cases of purulent meningitis in adults with L m were studied, and in four of these an

fixation test Only in two of these six cases is there a simultaneous increase of both antibodies These patients had all received intensive chemotherapeutic and antibacterial treatment in the acute stages of their illness It may reasonably be presumed that such therapy resulted in a less effective antigenic stimulation of the organs responsible for antibody formation so that the rate of immunization is markedly reduced in comparison with that in mothers of children with listeriosis

The Antibody Curves

Fig 1 illustrates the development of the antibody titres in 9 mothers of Listeria-infected foetus or child As might have been supposed a well-defined antibody curve is obtained In its later stages the antibody curve falls off very slowly proving the elevated titres to persist for several months In the acute phase on the other hand, the titre rises rapidly as evidenced by cases 6, 8, and 11 which also serve to exemplify the typical features of the later part of the antibody curve In cases 11 and 13 we may see the tendency for the complement fixation titre to increase later than the agglutination titre But in all important respects these antibody curves possess the same characteristics as those found in other bacterial diseases, although the comparatively slower retardation should be noted Since most of these mothers did not receive antibacterial therapy it may be suspected that the antigen was allowed to act for a considerable period of time thus sustaining the elevated antibody titre

Only three of the adult meningitis cases are useful for illustrative purposes and are shown in Fig 2 Here the antibody curves are lower but the trend is similar to that of other bacterial diseases It seems

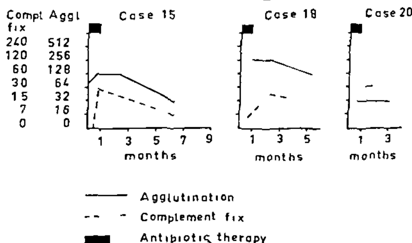


Fig 2

FROM THE INSTITUTE OF CLINICAL BACTERIOLOGY AND OF GYNAECOLOGY AND OBSTETRICS UNIVERSITY OF LUND GENERAL HOSPITAL MALMÖ SWEDEN

STUDIES OF ANTIBODIES IN HUMAN LISTERIOSIS

2 Antibody Titres in Healthy People and Patients of Different Categories

By

STEN WINBLAD and N E BORGLIN

Received 8.xii 62

If animals are immunized with Listeria monocytogenes (L m) they respond with elevated titres of O agglutinins and complement-fixing antibodies Also human cases of listeriosis show such an increase of the two types of antibody and this response is especially marked in women with newborns or abortions infected with L m (*Winblad*) It should be added that the antibody titre curves are best developed in the absence of antibiotic therapy

The possible existence of a serological cross reaction between agglutinins against L m and Staphylococcus aureus or enterococci (*Seeliger*) has caused some debate whether a high agglutination titre should necessarily be taken as specific proof of an infection with L m In the opinion of *Seeliger*, complement-fixing antibodies provide more valuable information

In the present investigation where O-antigens of the five different types (1 2, 3 4a, 4b) were used for agglutination reactions, and whole antigens of these strains were employed in complement fixation tests no evidence of any cross reaction has been noted with antigens of Staphylococcus aureus or enterococci (*Winblad*) Our findings are thus in agreement with those of *Welshimer*

It is important to find out if subclinical infections with L m are frequent and if they give rise to increased agglutination titres in comparatively healthy people If this should be the case, the responsible antibody is nevertheless to be regarded as "specific" In Germany, *Seeliger* found agglutination titres of 1 80 or more in 33 per cent of the human sera he examined In the same country, *Pöschel & Schwind* studied 504 healthy pregnant women and found agglutination titres of 1 60 in 24 2 per cent In consequence, these investigators consider high agglutination titres to be of diagnostic value only when associated with definite clinical symptoms

In USA, *Osbold & Sawyer* found an agglutination titre of 1/50 (for-

elevated O agglutination was found as well as an increased complement fixation titre

No cross agglutination against O antigen from Staphylococcus aureus or enterococci could be demonstrated with the methods employed However, the complement-fixing antibody may be the more specific one

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animals it seemed desirable to find out whether poultry farm workers have increased antibody titres Consequently, serum samples were collected from 30 such persons The result is presented in Table 1, showing that none of the persons of this study attain a positive or even weakly positive value Although the investigated material was not very large there seems to be no reason for suspecting contact with poultry to be a common cause of infection

TABLF 1

Distribution of Antibody Titres in Healthy Persons (in Per Cent)

Category	Total	O-Agglutina tion ≧64	O-Agglutina tion >64	Complement fixation ≧30	Complement fixation >30	O-Agglut =64 Cpl fix >30 or O-Agglut >64 Cpl fix =30 (+)	O-Agglut >64 Cpl fix >30 +	Column 5+6
Blood donors	240	13	1 7	22 8	9 5	2 1	0 8	2 9
Poultry farm workers	30	6 7	0	6 7	0	0	0	0

Distribution of Antibody Titres in Patients with Mononucleosis infectiosa and Non specified Diseases

With regard to Nyfeldt s early observation that an epidemic in Copenhagen of a disease closely resembling infectious mononucleosis might have been caused by L m, 48 sera delivered to the laboratory from patients with mononucleosis infectiosa and with a positive Paul-Bunnell's reaction were examined for antibodies against L m However, in these cases no positive or weakly positive titres were found ,Table 2) At the same time, examinations were made of 98 cases of pharyngitis showing clinical resemblance to mononucleosis infectiosa, but giving a negative Paul-Bunnel s reaction In none of these cases, either, was a positive titre to be found It is thus apparent that an infection with L m should not be regarded as commonly associated with mononucleosis infectiosa

Also recorded in Table 2 is an examination of sera from 100 different patients, treated at the department of internal medicine in Malmo These patients were suffering from various diseases but only 2 cases showed titres designed positive according to the criteria stated above A similar investigation was performed in a hospital situated in a rural area (Horby) where the population consists largely of farmers who to a large extent have very close contacts indeed with poultry 76 patients of this latter group were examined, and only in one case (1 3 per cent) was there an elevated titre, and this titre was encountered in a sailor who had just returned from tropical waters It is thus evident that not even this last group lends any support to the theory that a serologically verifiable contact with L m should be associated with persons in frequent contact with poultry

malinized antigen) in 5 3 per cent of normal subjects and consider a moderate titre of *e g* 1/25 to be so common as to be regarded 'normal"

As may be seen from our previous report, in cases of bacteriologically verified human listeriosis there are increased titres of O-agglutinins and complement-fixing antibodies especially in the absence of anti bacterial therapy Antibody curves are produced that are in full agreement *with those of other bacterial infections A new investigation there*fore seemed called for in which the same technique was employed but this time on a large material including both healthy subjects and patients suffering from various diseases It was hoped that such a study would throw light on the average amount of such antibodies in the Swedish population and also on the frequency with which the disease tends to appear in Sweden

METHODS

The preparation of antigens and the techniques of the O agglutination and complement fixation reactions have been described in the first part of this study (*Hinblad*)

Sera from healthy and diseased subjects tabulated below in connection with the results obtained were examined after inactivation at 56° C for 30 minutes

RESULTS

Distribution of Antibody Titre in Healthy Persons

Blood donors are taken here to represent healthy individuals, since, in order to be accepted as donors, they are subjected to ESR and Wassermann tests, and the state of their blood and lungs is controlled Table 1 shows that among 240 such blood donors, 87 per cent had an agglutination *titre of less than 1/64 11 3 per cent* had a titre of 1 64 and 1 7 per cent had a higher titre As for complement-fixation, 77 2 per cent had a titre of less than 1/30, 13 3 per cent a titre of 1/30 and 9 5 per cent had a higher titre In consideration of this distribution it seems justified to regard O-agglutination titres higher than 1/64, and complement-fixation titres higher than 1/30 as sufficiently increased, compared to normal conditions, to be taken as indicative or of a recent or actually present contact with L m A combination of elevated titres in both reactions is marked out with a positive sign in the table, whereas an increased titre of either antibody combined with the limit value of the other is considered weakly positive On this assumption, 2 9 per cent of the blood donors are found to have an increased antibody titre against L m

The frequency of Listeria monocytogenes in animals sent in for autopsy to the State Veterinary Institute of Sweden has been reported by *Nilsson & Karlsson* In the years 1948 1957 they found these bacteria in 141 instances, and of these no less than 111 were poultry Although this high contribution on the part of the poultry may possible be due to the fact that they are more frequently sent in for autopsy than other

first of the groups tabulated here comprises cases where the pregnancy took a normal course and the baby showed no signs of illness when leaving the maternity clinic The investigation was started after the occurrence of a few cases of listeriosis and includes 2 271 successive maternity cases, no selection attempted Throughout this whole investigation, no single case of bacteriologically verified listeriosis was found in the clinic It will be seen from the table that the percentage of cases showing an elevated antibody titre is small (1 1 per cent) being closely similar to the previously studied normal material In spite of this low percentage, though, it should be noted that there are mothers who have an elevated titre but who nevertheless showed no signs of illness and gave birth to apparently healthy children As was the case with the previously described normal material it must be supposed that such an elevated titre may be the result of a sub-clinical infection at a date that cannot be determined No antibiotic therapy was given on the strength of these findings alone, and the pregnancy was allowed to take its normal course There is thus no reason to suspect that a newborn child is going to have listeriosis merely because its mother shows an elevated antibody titre Another interesting thing that emerged from this investigation was the fact that several of these women who had an elevaed antibody titre were immigrantes from Central Europe Possibly the infection is more common in other European countries than in Sweden where after all, the risk seems comparatively small

86 cases of imminent abortion were examined, too, but none of these showed antibody titres higher than the supposed limit values

The third group consists of 102 non-specified abortions—that is, there was no special selection, every woman who happened to have an abortion during the period of investigation was subjected to a serological examination Within this group, there is a weak but no significant tendency for the women to have higher antibody titres than the large group of normal pregnant women

The next three groups recorded in Table 3 are "selected", however, These sera do not originate from the Malmö clinic but were obtained from other Swedish maternity clinics where some obstetrical complication had occurred and an infection, *e g* listeriosis, seemed a conceivable explanation As distinct from the first three groups of this material where entirely nonselective principles were abided to, these three groups may be said to have been specially chosen on the suspicion of a possible infection Within this last group of abortions, a considerable number of the women [illegible] ated antibody [illegible] h L m Of the [illegible] cent have an [illegible] titre, and the mothers of diseased or deformed children have increased titres in 12 9 per cent There is obviously a much higher percentage of elevated antibody titres in these groups than in the normal material It is probable that in many of these cases listeriosis

TABLE 2

Distribution of Antibody Titres in Patients with Mononucleosis infectiosa and Non specified Diseases (in Per Cent)

Patient category	Total	O Agglutination		Complement fixation		O Agglut =64 Cpl fix <30 or O Agglut >64 Cpl fix =30 (+)	O Agglut >64 Cpl fix >30 +	Column 5 + 6
		≤64	>64	≤30	>30			
Mononucleosis infect (Positive Paul Bunnell reaction)	48	27.8	6.3	14.6	6.3	0	0	0
Pharyngitis (Negative Paul Bunnell reaction)	98	7.1	1.0	11.2	6.1	0	0	0
Non specified disease (City of Malmo)	100	8	0	6	4	2	0	2
Non specified diseases (Rural district) (Horby)	76	13.8	2.6	11.8	9.2	1.3	0	1.3

TABLE 3

Distribution of Antibody Titres in Obstetrical Patients in Various Situations (in Per Cent)

Patient category	*Total*	O-Agglutination		Complement fixation		O Agglut =64 Cpl fix 30 or O Agglut >64 Cpl fix 30 (+)	O-Agglut >64 Cpl fix >30 +	Column 5 + 6
		≤64	>64	≤30	>30			
Normal pregnancies with healthy offspring	2271	17.2	4.7	8.5	3.5	0.2	0.9	1.1
Imminent abortions	86	15.1	2.3	2.3	0	0	0	0
Abortions (non specified Malmo)	102	19.1	7.8	8.8	9.9	1.9	1.9	3.8
Abortions (unknown causes suspected infections)	121	43.5	20.5	29.7	12.4	10.7	5.8	16.5
Mothers of children dead in the perinatal period	158	30.7	12.4	17.8	7.7	4.8	3.6	8.4
Mothers of diseased or deformed children	85	34.4	19.5	22.3	8.2	8.2	4.7	12.9

Antibody Titres of Obstetrical Patients in Various Situations

Since listeriosis plays an important part especially among the diseases of newborn children it seemed desirable to establish the frequency of elevated antibody titres in normal pregnancies as well as various obstetrical complications, such as abortion imminent abortion death of the child in the perinatal stage or illness during the neonatal period The result of such an investigation is set forth in Table 3 The

is combined with a complement fixation titre above 1/30 or a complement fixation titre of at least 1/30 with an O agglutinin titre above 1/64

There is no evidence from this investigation that persons occupied with poultry farming or otherwise belong to the rural population have higher antibody titres than other people The road which L m may take from animal to human hosts is still unknown

Nyfeldt's proposition that mononucleosis infectiosa might have some relation to an infection with L m was in no way substantiated by our results Nor is there any reason to believe that cases of pharyngitis of similar descriptions but with a negative Paul Bunnell's reaction have any connection with L m

In cases of uncomplicated pregnancies in our country there seems to be no greater frequency of increased antibodies against L m than in other persons (2–4 per cent)

On the other hand elevated antibody titres are comparatively frequent (8 16 per cent) in women whose pregnancies have been complicated by abortions of unknown aetiology or perinatal death of the children or whose newborns have been diseased or deformed from presumably infectious causes Here an infection with L m is often a possible explanation It should be kept in mind however, that 2–4 per cent may have elevated titres but show no clinical signs of listeriosis Such an elevated titre is therefore no proof of actual clinical listeriosis but should nevertheless be noted Pregnant women with increased titres and the newborn children of such mothers should also be kept under close observation since any signs of meningitis on the part of the child urge immediate antibiotic therapy

The previous reported material containing sera from diagnosed cases of human listeriosis also shows that antibodies will develop if no early antibiotic treatment is given Furthermore it proves serological findings to be good evidence of recent or actually present infections with L m

SUMMARY

Healthy subjects and groups of patients suffering from different diseases were examined for elevated titres of antibodies against L m O agglutinin titres above 1/64 and complement fixing antibodies in titres above 1/30 are regarded as significantly increased and suggestive of an infection with L m Such an elevated value in combination with the limit value of 1/64 in the case of O agglutination or 1/30 in the case of complement fixation is interpreted in a similar manner

In 240 healthy blood donors elevated titres occurred in 2.9 per cent In 48 cases of mononucleosis infectiosa there was no single case of any elevated antibody titre against L m Nor could any elevated titres be demonstrated in 98 patients with pharyngitis but with a negative Paul Bunnell's reaction In patients suffering from various internal di

lies behind the abortion or the perinatal complication It is not intended to analyze individual cases here, that will be done in a later report

It will be realized from what has just been said that listeriosis seems a reasonable explanation of several cases of obstetrical complications with abortions and perinatal deaths As a consequence, it is important for diagnostic purposes to make cultivations on material from the vagina, abortions, and dead infants, and to study the course and type of the antibody curves in the mothers

Antibody Titres in Cases of Human Listeriosis

Previously reported, diagnosed cases of listeriosis (*Winblad*) are mentioned here for comparison with the material described above In mothers of newborns or abortions, the frequency of elevated antibody titres calculated according to the criteria used in this study, amounts to 88 per cent Although the number of cases examined is not very large it warrants the conclusion that elevated antibody titres accompany the infection In these mothers no antibiotics were administered at the early stage of the obstetrical complication, and for this reason the development of antibodies remained unaffected In the case of meningitis caused by L m, on the other hand, antibiotic treatment was started very soon and this probably explains why only 1/3 of these cases have an antibody titre higher than the calculated limit values

DISCUSSION

Clinical infections with Listeria monocytogenes occur in Sweden, although they are relatively rare However, titres of O-agglutinins below 1/64, and complement-fixing antibodies below 1/30 are by no means uncommon in healthy people in this country Only in 2–4 per cent though, are these limit values exceeded, that is an O-agglutination titre of 1/64 in combination with a complement-fixation titre of more than 1/30, or a complement-fixation titre of 1/30 in combination with an agglutination titre of more than 1/64 If we regard these combinations as typical of a recent or actually present infection with L m, such elevated titres can only be demonstrated in those 2–4 per cent It is an open question whether titres lower than those mentioned are indicative of a cross immunity against other antigens among Gram-positive bacteria or whether they are evidence of a subclinical infection with L m Provided that the titres are not a manifestation of any cross antigenicity the conclusion must be that latent and subclinical infecions with L m are comparatively common but do not usually lead to any considerable increase of antibodies This finding, however, applies only to Sweden, and in all likelihood similarly performed investigations in South or Central Europe might show elevated antibody titres to be much more frequent As things stand however, the result is possibly indicative of a clinical infection with L m if an O-agglutinin titre of at least 1/64

THE DEPARTMENT OF BACTERIOLOGY (HEAD PROF R GRUBB) AND THE DEPARTMENT OF PATHOLOGY (HEAD PROF C G AHLSTRÖM), UNIVERSITY OF LUND, SWEDEN

TRIALS WITH A RAPID METHOD FOR QUANTITATIVE AND QUALITATIVE VIRUS DETERMINATION BY MICROSCOPIC EXAMINATION OF THE INDIVIDUAL CELLS IN VIRUS INOCULATED MONOLAYERS ON SLIDES

By

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Microscopic examination of the effect of viruses on cells is generally performed on monolayers cultivated on coverslips or on squashed cells Recently, a simple method was devised for cultivating monolayers on slides in Hellenthal cuvettes (*Bergman* 1959) Slides are easy to handle and the possibilities of examining the individual cells are good The procedure therefore appears to enable a rapid and simple estimation of a virus sample inoculated on a monolayer A preliminary report of a trial with such a method is given below

MATERIAL AND METHODS

Mediums TCM [illegible]

Cells Human amniotic cells prepared according to *Lahelle* (1956) All cells used in one experiment were from the same placenta.

Viruses Adenovirus type 7 Pol [illegible] were supplied by the State Serum Ins[illegible] he viruses are given in the Tables All [illegible]

Experimental procedu[illegible] was fixed on a slide by [illegible] of cell suspens[illegible] th[illegible] su[illegible] be[illegible] sc[illegible] ac[illegible] in Tables [illegible] experiments [illegible] cells and viruses were added to the slides simultaneously

Attachment of the cells and adsorption of the viruses took place with the slides horizontal in moist air with 8 per cent CO_2 in a covered chamber at 37° C After

The investigation was supported by grants from the *Swedish Medical Research Foundation* and the *Medical Faculty of Lund*

seases, an elevated antibody titre against L m was found in 2 per cent and in a rural population, similarly examined, in 1 3 per cent Persons dealing with poultry do not have antibody titres against L m higher than normal

In normal pregnancies as well as in a series of undifferentiated abortions, an elevated antibody titre was demonstrated in 1 1 per cent only In a selected material of abortions from unknown, but probably infectious causes, or of mothers of infants that died in the perinatal period or of mothers with diseased or deformed children, however, the amount of serologically positive cases was considerably increased (8 16 per cent), and in these last groups it must be supposed that some of the obstetrical complications were caused by L m

Healthy pregnant women who gave birth to living, healthy babies showed the same percentage of increased antibody titres as other healthy subjects An infection with L m although stimulating a development of antibodies may thus not always produce any clinical disorder in the child Also, the infection indicated by the elevated antibody titre may belong to the past

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TRIALS WITH A RAPID METHOD FOR QUANTITATIVE AND QUALITATIVE VIRUS DETERMINATION BY MICROSCOPIC EXAMINATION OF THE INDIVIDUAL CELLS IN VIRUS INOCULATED MONOLAYERS ON SLIDES

By

SVEN BERGMAN, INGRID STENRAM and UNNE STENRAM

Received 13 xi 62

Microscopic examination of the effect of viruses on cells is generally performed on monolayers cultivated on coverslips or on squashed cells Recently, a simple method was devised for cultivating monolayers on slides in Hellenthal cuvettes (*Bergman* 1959) Slides are easy to handle and the possibilities of examining the individual cells are good The procedure therefore appears to enable a rapid and simple estimation of a virus sample inoculated on a monolayer A preliminary report of a trial with such a method is given below

MATERIAL AND METHODS

Mediums TCM 199 with 20 per cent pooled human inactivated serum or 20 per cent rabbit serum or [illegible]
penicillin ml and 100 [illegible]
for maintenance med [illegible]
against adenovirus 7 [illegible]
expressed as serum d [illegible]
100 TCD_{50} of virus

Cells Human amniotic cells prepared according to *Lahelle* (1956) All cells used in one experiment were from the same placenta

Viruses [illegible]
by the Sta [illegible]
given in th [illegible]
Experim [illegible]
was fixed on a slid [illegible]

[illegible] of the cells and adsorption of the viruses took place with the slides horizontal in moist air with 8 per cent CO_2 in a covered chamber at 37° C. After

The investigation was supported by grants from the *Swedish Medical Research Foundation* and the *Medical Faculty of Lund*

seases, an elevated antibody titre against L m was found in 2 per cent and in a rural population, similarly examined, in 1 3 per cent Persons dealing with poultry do not have antibody titres against L m higher than normal

In normal pregnancies as well as in a series of undifferentiated abortions, an elevated antibody titre was demonstrated in 1 1 per cent only In a selected material of abortions from unknown, but probably infectious causes, or of mothers of infants that died in the perinatal period or of mothers with diseased or deformed children, however, the amount of serologically positive cases was considerably increased (8 16 per cent), and in these last groups it must be supposed that some of the obstetrical complications were caused by L m

Healthy pregnant women who gave birth to living, healthy babies showed the same percentage of increased antibody titres as other healthy subjects An infection with L m although stimulating a development of antibodies may thus not always produce any clinical disorder in the child Also, the infection indicated by the elevated antibody titre may belong to the past

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various intervals (given in the Tables) the fluid was pipetted off and the rings were removed The slides were carefully washed in medium as a rule the one containing 20 per cent pooled human inactivated serum and placed vertically into Hellenthal cuvettes containing this medium Each cuvette accommodated 23 slides The loaded cuvettes were placed in moist air with 8 per cent CO_2 at 37° C

Check examination of the supernatant and the monolayers revealed that 80–95 per cent of the deposited cells had attached to the slides

Cytological methods Slides were removed from the cuvettes at different intervals (see the Tables) washed in physiological saline (37° C) for 3 minutes and fixed in

t non specific changes (cf *Dunnebacke* 1956) In the early stages of specific cell degeneration caused by poliomyelitis virus type 3 (Fig 3) the nuclei were wrinkled and the nucleoli could often not be identified In these stages the cytoplasm was still of normal outline though it was often vacuolized and sometimes contained basophil granules Sometimes the cytoplasm showed small spherical projections In later stages in which the changes could not be distinguished from non specific degeneration the cells were rounded and showed no such projections while the

ner phery f the

under a × 10 c

examined Those [illegible] cytopathic changes were then seen were marked 0+ in the tables When the cells had become attached to the sl[illegible]

[illegible] cultures

examination of a slide required 5 10 minutes

EXPERIMENTS OBSERVATIONS AND DISCUSSION

Adsorption of Viruses

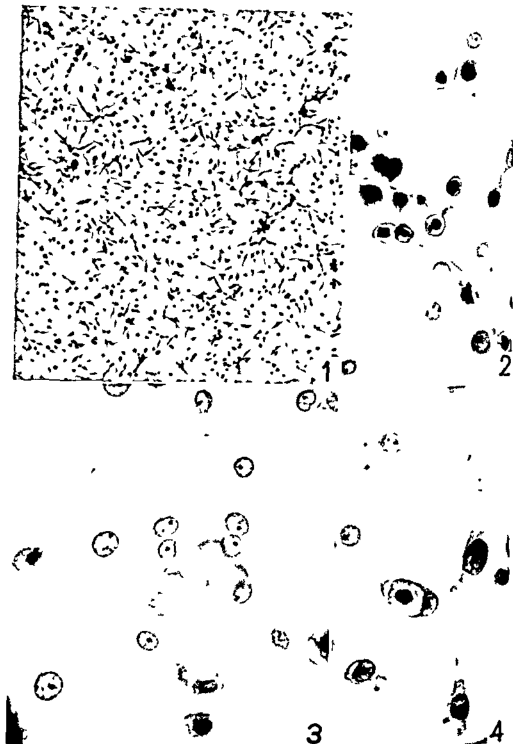
The adsorption experiments were carried out with amniotic cells which were allowed to attach to the slides for 1 hour The preparation was then inoculated with viruses which were given ½, 2 or 6 hours to adsorb to the cells The results are given in Tables 1 and 2

Fig 1 Control culture 4th day × 58

Fig 2 Culture inoculated with adenovirus type 7 4th day 10 cells with specific cytopathic changes are seen in focus × 420

Fig 3 Culture inoculated with poliomyelitis virus type 3 2nd day 7 cells with specific cytopathic changes are seen in focus × 420

Fig 4 Control culture 2nd day Centrally a cell with non specific degeneration × 420



Figs 1–4
Cultures of human amniotic cells fixed in 96 per cent alcohol and stained with Htx eosin

amniotic cells within 6 hours but not within 2 hours. This assumption is in good agreement with the findings of *Ginsberg* (1958 a), *Lieberman* & *Friedman* (1959), and *Pereira* (1959). The absence of any effect of the supernatant fluid after an adsorption period of 6 hours might also be explained by the assumption that viruses not adsorbed within this period are inactivated. However, tests in which the primary virus material was allowed to be "adsorbed" for 6 hours to slides without cells still showed full activity in the supernatant.

TABLE 3

Amount of Viruses Adsorbed after Various Time Intervals

Adenovirus Type 7. Titre log TCD 50 0.1 ml 4.0. 100 000 Cells per Slide

	2 h	supern.	total	6 h	supern.	total
A	376	432	808	680	0	680
	458*	531	989	736	1	737
B	343	373	716	743	0	748

Read on sixth day.
In "A" the cells were allowed 1 hour to become attached to the [illegible]

TABLE 4

Amount of Viruses Adsorbed after Various Time Intervals

Poliomyelitis Virus Type 3. Titre log TCD 50 0.1 ml 3.5. 100 000 Cells per Slide
See also Table 3

	2 h	supern.	total	6 h	supern.	total
A	42	114	156	90	23	113
	44	113	157	112	21	133
B	14	98	[illegible]	117	13	130

Read on sixth day.

On comparison of the data given in Tables 1 and 3 it will be seen that a difference was found between the effect of the virus after 2 hours and 6 hours, respectively, in the experiments accounted for in Table 3 but not in the experiments summarized in Table 1. The virus dose used was the same in both experiments. In the one experiment (Table 1) the number of cells was 60 000 per slide as against 100 000 in the other (Table 3). The amniotic cells used in one experiment originated from one placenta and those used in the other from another placenta. This may help to explain the difference mentioned, as it is known that the susceptibility of amniotic cells to viruses may vary from one placenta to another (*Osterhout* & *Tamm* 1959). It should also be remembered

TABLE 1

Adsorption Time of Viruses

Adenovirus Type 7, Undiluted Titre log TCD 50/0 1 ml–4 0 60 000 Cells per Slide
Table Gives Number of Cells per 1,000, with Specific Cytopathic Changes

Adsorption time in hours	*Interval in hours after commencement of inoculation* 20	45	68	93
½	1	183	279	311
2	5	405	695	607
6	4	490	503	689

TABLE 2

Adsorption Time of Viruses

Poliomyelitis Virus Type 3 Undiluted Titre log TCD 50 0 1 ml –4 6 60 000 Cells per Slide Table Gives Number of Cells per 1 000, with Specific Cytopathic Changes

Adsorption time in hours	*Interval in hours after commencement of inoculation* 20	45	68	93
½	24	83	130	52
2	44	252	222	262
6	275	680	677	741

As regards adenovirus type 7 (Table 1), the number of cells with specific cytopathic changes increased slightly on prolongation of the adsorption period from 2 to 6 hours When the virus had been allowed only ½ hour for adsorption, the effect was considerably lower The effect of poliomyelitis virus type 3 increased with the adsorption time (Table 2)

To test how complete virus adsorption was after 2 and 6 hours, supernatant fluid was pipetted off at these intervals The fluid was added to suspensions of cells from the same population as that used in the primary test The cells were allowed to attach on slides After 6 hours the supernatant was pipetted off, and the slides were washed in medium with 20 per cent pooled human inactivated serum and placed in Hellenthal cuvettes containing this medium The cuvettes were handled as described above At different intervals the slides were taken for cytological examination The results are given in Tables 3 and 4

The results in Table 3 show that primary adsorption of adenovirus type 7 for 6 hours but not for 2 hours gave a supernatant fluid, which, as judged by the same method on new slides was completely or almost completely free of infectious viruses The sum of the effect caused by the primary virus sample after an adsorption time of 2 hours and its supernatant fluid was nearly the same as the virus effect of 6 hours' primary adsorption The findings suggest that under the conditions used adenovirus type 7 is completely or almost completely adsorbed to

amniotic cells within 6 hours but not within 2 hours This assumption is in good agreement with the findings of *Ginsberg* (1958 a), *Lieberman & Friedman* (1959), and *Pereira* (1959) The absence of any effect of the supernatant fluid after an adsorption period of 6 hours might also be explained by the assumption that viruses not adsorbed within this period are inactivated However, tests in which the primary virus material was allowed to be "adsorbed" for 6 hours to slides without cells, still showed full activity in the supernatant

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Read on sixth day

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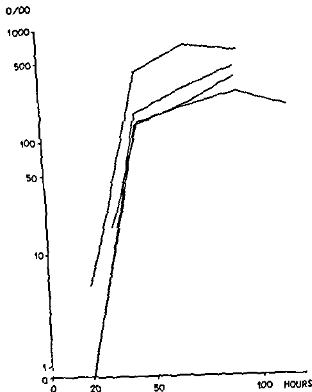


Fig 5

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are accounted for

(cf under Material and Methods) Non-specific cell degeneration was seen in the controls generally in about the same percentage as in high virus dilutions

Titration of Virus

Experiments were performed with poliomyelitis virus type 3 and adenovirus type 7 in various dilutions to study a possible quantitative relationship between inoculated virus and the number of cells with specific cytopathic changes (see Tables 5 and 6)

The tube technique is more sensitive than the slide technique at high virus dilutions That is certainly due to the fact that attack on a single cell in the tube results in the breakdown of the whole culture according to the all or-none principle If secondary spread of viruses is restrained, as seems to be the case with our slide technique (see below), the attack

that not all cells in a population are susceptible to viruses (*Dulbecco* 1958, and others) When, therefore, as in the experiments accounted for in Table 1, the virus produces a strong effect already after 2 hours adsorption, the effect can hardly be expected to increase much on increase of the dose or extension of the adsorption period

Poliomyelitis virus type 3 had stronger effect after an adsorption period of 6 hours than of 2 hours, but after both periods the supernatant still contained infectious viruses (Table 4) This was presumably due to incomplete adsorption of viruses Previous investigators have also found that if poliomyelitis virus is allowed 1 hour in which to be adsorbed to monkey kidney tissue cells and the cells are then thouroughly washed, poliomyelitis virus will still be demonstrable in the medium during the first few hours (*Reale et al* 1956, *Reissig et al* 1956) A release of new-formed poliomyelitis virus and an increase in the virus titre of the medium has been demonstrated for HeLa cells 6 hours after inoculation (*Lwoff et al* 1955), for monkey kidney cells 5 6 hours after inoculation (*Reale et al* 1956, *Reissig et al* 1956) but not until 8 hours after inoculation for amniotic cells (*Dunnebacke* 1956) Since amniotic cells were used in the present investigation and since the poliomyelitis virus activity in the supernatant was lower after an adsorption time of 6 hours than of 2 hours, it is probable that no more than a negligible amount of new-formed viruses were released during the period of adsorption

Interval between Inoculation and Reading of Result

The cultures were examined at various intervals after inoculation but not later than on the seventh day Adenovirus type 7 was found to produce cytopathic changes in only a few cells within the first 24 hours On the second day, however, the changes in the cell cultures were marked There was a slight increase in the number of affected cells on the third day The extent of the alterations persisted unchanged on the fourth day (Tables 1 and 5 and Fig 5)

The tests with poliomyelitis virus are given in Tables 2 and 6 and Fig 6 As mentioned under Material and Methods the changes caused by the virus could not always in late stages be distinguished with certainty from non specific cell degeneration The numbers of cells showing signs of non-specific degeneration are therefore included in brackets in Table 6 It is clear from the table that the number of cells with specific cytopathic changes was fairly constant from the second to the fifth day inclusive

It is also seen in Table 6 that the number of cells with changes of unspecific appearance was largest in the slides inoculated with virus in high concentrations The explanation is probably that some of the cells showing apparently unspecific changes were in fact degenerating owing to virus infection, though that could not be recognized in *late* stages

TABLE 5

Effect of Virus Dilution

Adenovirus Type 7 Titre log TCD 50 0 1 ml –4 0 100 000 Cells per Slide Adsorption Time 2 Hours Table Gives Number of Cells per 1 000, with Specific Cytopathic Changes

Experiment	Virus dilution	Interval in days after commencement of inoculation 1	2	3	4	5	6
A	10^{0}	0	139	214	360		
	10^{-1}	0	49	51	117		
B	10^{0}	0	175	301	442		
	10^{-1}	0	60	57	104		
C	10^{0}	16	143	205	262	193	–
	10^{-1}	1	1	12	13	23	13
	10^{-2}	0	0	1	0+	3	0+
	10^{-3}	0	0	0	0	2	0+
	10^{-4}	0	0	0	0	0	0

TABLE 6

Effect of Virus Dilution

Poliomyelitis Virus Type 3 Titre log TCD 50 0 1 ml –3 5 100 000 Cells per Slide Adsorption Time 6 Hours Table Gives Number of Cells per 1,000, with Specific Cytopathic Changes Bracketed Figures Indicate Number of Cells, per 1 000, with Non Specific Changes

Virus dilution	Interval in days after commencement of inoculation 1	2	3	4	5	6	7
10^{0}	32(29)	47(38)	50(16)	64(18)	64(26)	51(93)	43(102)
10^{-1}	0+(17)	1(6)	4(2)	4(5)	8(12)	9(12)	0(75)
10^{-2}	0(17)	0+(7)	0(6)	0+(10)	1(3)	0+(6)	3(21)
10^{-3}	0(5)	0(9)	0(4)	0(2)	0+(4)	0(4)	0(5)
10^{-4}	0(9)	0(1)	0(4)	0(8)	1(9)	0(6)	0(67)
10^{-5}	0(19)	0(5)	0(2)	0(2)	0(6)	0+(16)	0(59)
Controls	0(12)	0(8)	0(7)	0(9)	0(2)	0(11)	0(33)

The differences between experiments A, B and C in Table 5 are probably explained by the different virus susceptibility of amniotic cells from various placentas (*Osterhaut & Tamm* 1959)

The results with our slide technique (see Figs 5 and 6) indicate that no major spread of viruses occurred during the observation periods That was certainly due to the medium employed, pooled human serum, which contained antibodies and, may be, other substances inhibiting poliomyelitis virus type 3 and adenovirus type 7 When rabbit normal serum was employed in the maintenance medium there was a stronger effect on the culture, especially in the experiments with poliomyelitis virus type 3, indicating secondary spread of viruses (unpublished experiments) To obtain a true relationship between the number of affected cells and deposited infectious virus particles there should be no

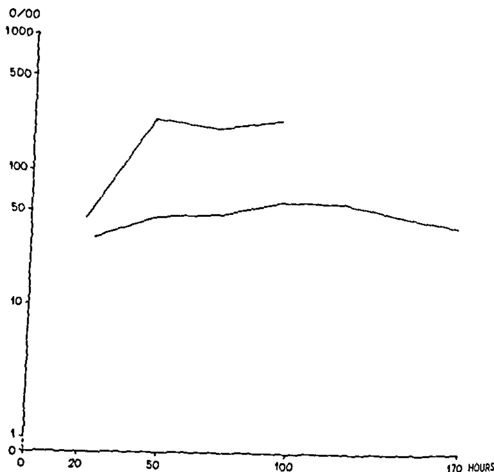


Fig 6

Poliomyelitis virus type 3 The Fig gives in log scale number of cells per 1000 with specific cytopathic changes Each line represents a separate experiment Virus titre in the experiment of the upper line log TCD 50 0 1 ml 4 6 and adsorption time 2 hours Virus titre in the experiment of the lower line log TCD 50 0 1 ml -3 5 and adsorption time 6 hours The experiments are accounted for Tables 2 and 6

on a single cell must be difficult to detect Another factor which may be of importance for the sensitivity is that in the tube technique the monolayer is examined microscopically before inoculation with the virus Cell cultures in a good condition are selected and this might influence the number of cells attacked No such selection is possible in the slide technique if the virus and the cells are deposited on the slides simultaneously

Dilution of poliomyelitis virus (Table 6) as well as high dilution of the adenovirus (Table 5, exp C) was followed as expected, by about tenfold reduction in the number of cells showing specific cytopathic changes In the experiments using large doses and producing marked effects (Table , exp A and B), the effect of the dilutions was inferior to the effects expected from the thus reduced number of viruses inoculated As mentioned, however not all cells in a cell population are susceptible to viruses

Examination of a single slide within 1–3 days is often sufficient to estimate the titre of the virus The slide technique, as here employed, is, however, not suitable for titration of viruses which spread from cell to cell without passing through the medium or which form multinucleated cells

The slide technique is less sensitive than the tube technique at low virus concentration, probably because the attack on a single cell must be difficult to detect on a slide

SUMMARY

A simple method is described for cultivating viruses in monolayers on slides Adenovirus type 7 and poliomyelitis virus type 3 have been tested Pooled human serum was employed in the maintenance medium The effect of the virus is studied by microscopical examination of the individual cells The effect of variation in the time of virus adsorption and of the interval between inoculation and reading of the results was studied The results suggest that the method can be used for a rapid ascertainment of a virus attack and, at least for some viruses, in the estimation of their titre

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secondary spread of viruses in the cultures It must be examined for every virus studied if this necessitates the addition of specific antibodies to the medium Viruses which spread from cell to cell without passing through the medium or which form multinucleated cells are probably not suitable for titration according to the slide technique as here employed

When trying to estimate the titre of a virus sample by the slide technique a few more points must be considered *The slide must be examined at a time when all affected cells show specific cytopathic changes and none of these has been lost* The number of cells deposited on the slides should not change during the incubation period otherwise the *percentage of affected cells will not be constant*

Summing up, it may be concluded that an advantage of titration with our slide technique is that it does not require end-point dilution *Determination of the percentage of altered cells on a single slide at a suitable time may be sufficient to estimate the titre of the virus*

GENERAL COMMENTS

Judging from our experience with the slide technique described for cultivation and titration of viruses the following points deserve comment

Attempts were made to provide optimal environment for the cells Human serum was therefore employed in the maintenance medium The occurrence of antibodies or other inhibitory substances in human serum (see for example *Ginsberg* 1958 b) did not prevent the appearance of specific cytopathic changes but it may prevent secondary spread of the virus within the cultures The washing of the slide after adsorption of the viruses diminishes the non-specific toxic effects, *e g* of a faecal sample

Several slides can be placed in the same cuvette This eliminates differences in pH, temperature, composition of the medium etc between *different cultures of one and the same experimental series* It saves cells and space

As here employed, the slide technique requires, in contrast to the tube technique, staining of the specimens and cytological examination under a high power microscope Furthermore, many viruses produce readily recognizable changes in the cells With the slide technique it is therefore in many cases possible to ascertain whether or not the cells have been attacked by viruses and sometimes to form an idea of the group of virus within 1–3 days

In contrast to the tube and plaque techniques, using the slide technique it is not necessary to wait until the cells have been broken down with secondary spread of the virus to the environments The difficulties in differentiating between specific and unspecific cell degeneration must be carefully evaluated for every virus concerned

ment of different pictures of disease for instance kidney tumours or anaemia instead of leukaemia

The intracellular relationship between the virus and the host has been sudied with morphological biochemical and biophysical techniques The intra cytoplasmatic localization of the active virus as well as the biochemical composition and activity of the virus particle connects it to the nucleotide metabolism of the cytoplasm Quantitative alterations in the cellular nucleotide metabolism corresponding to the enzymatic pattern of the virus particle can be demonstrated These alterations might be related to changes in both the energy metabolism and the polynucleotide synthesis of the leukaemic cell

Analyses of the base composition of the viral ribo nucleoprotein reveal possibilities also for an "epigenetic" action of the virus within the cell This action, however does not seem to affect species specific properties of the leukaemic cell for instance its type of haemoglobin or its chromosome morphology

Ahlstrom C G Institute of Pathology University of Lund Lund ROUS SARCOMA IN MAMMALS AND DUCKS

Diderholm H & Wesslen T Institute of Virology University of Uppsala Uppsala INHIBITION OF POLYOMA VIRUS HAEMAGGLUTINATION BY DIFFERENT TISSUES OF MOUSE AND HAMSTER

A study was made on the interaction between polyoma virus and different tissues of newborn and adult mouse and hamster Homogenates prepared from a number of different organs were found to inhibit polyoma virus haemagglutination Homogenates of salivary glands of newborn mouse were most effective (haemagglutination inhibition titre of 1/8192) Also homogenates of brain kidney and adult salivary glands inhibited haemagglutination in high dilutions (1/512 to 1/1024) Low titres or no inhibition at all were found with homogenates of most other organs

Homogenized cells had a higher capacity than intact cells to inhibit haemagglutination Most of the inhibitory substance was found in the microsomal fraction It was found to be sensitive to RDF and periodate but not to trypsin, chymotrypsin, lipase ether and chloroform

The question was discussed to what extent the inhibitory substance consists of a receptor substance to polyoma virus and the influence it may have on the tumour formation

Meeting December 1

Bottiger Margareta National Bacteriological Laboratory Stockholm ANTIBODIES AFTER POLIO VACCINATION

The antibody status before during and after vaccination with inactivated polio vaccine has systematically been studied in Sweden since 1957 Discrepancies both quantitative and qualitative were observed between the three different types of polio Constantly high and stable titres were found against type 2 Against type 1 the antibody response was considerably lower and the antibodies showed a higher tendency to decrease Against type 3 the primary response except in 1957, showed high values equal to those obtained by type 2 These antibodies though showed the same unstable character as those against type 1

A control in 1961 of the vaccinees 1957 thus revealed considerably decreased anti

TRANSACTIONS OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

Annual Meeting November 30 and December 1, 1962

Stockholm, Sweden

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Meeting November 30

PANEL DISCUSSION ON TUMOUR VIRUSES

(S Gard Moderator)

Klein G Institute of Tumour Biology Karolinska Institutet Stockholm A SHORT SURVEY OF SOME MAIN PROBLEMS IN MODERN EXPERIMENTAL TUMOUR VIROLOGY

Sjogren O Institute of Tumour Biology Karolinska Institutet Stockholm SPECIFIC CELLULAR ANTIGENS IN VIRUS INDUCED TUMOURS

There is now indications for the presence of tumour specific antigens in tumours induced with polyoma virus SV40 virus Moloney virus Gross virus Shope virus and Rous virus The specific cellular antigenicity appears to be common for all tumours induced with the same virus but differs in tumours induced with different viruses This is in contrast to the finding that tumours induced with chemical carcinogenesis possess an antigenicity specific for each individual tumour The implications of these findings for tumour therapy or prophylaxis are that a specific immunological prophylaxis is ruled out in nonviral tumours because of the absence of common specific antigens but might be possible with certain limitations with virus induced tumours

Oker Blom N Institute of Virology University of Helsinki Helsinki Finland INTERFERENCE PHENOMENA WITH TUMOUR VIRUSES

Thorell B Institute of Pathology Karolinska Institutet Stockholm RELATIONSHIP OF VIRUS AND CELL IN EXPERIMENTAL LEUKAEMIA

The formation and development of leukaemic cells from the virus infected target cells in the haemopoietic organ has been studied with cytological as well as quantitative cytochemical methods The leukaemic cell development is described in comparison to the normal blood cell formation and is characterized *inter alia* by accumulation of cytoplasmic ribo nucleoprotein

The ability of the leukaemia virus to affect other types of target cells than the bone marrow elements depends on the host reactions for instance tolerance or antibody formation Different host reactions in this respect can lead to the devel p

human serum totally—to regenerate the inhibitory effect of the serum Some of the sera were tested against adenovirus types 1 2 3 5 and 7 A as well A difference between otherwise identical samples before and after heat inactivation was recorded only with type 7 in these cases The other types tested including type 7 A (S 1058) were not influenced in this way

Hedstrom C E & Lycke E Municipal Virological Laboratory and the Virological Laboratory of the Department of Bacteriology University of Gothenburg Gothenburg AN EXPERIMENTAL STUDY OF OYSTERS AS VIRUS CARRIERS

Epidemics of hepatitis spread by raw oysters have been described from Sweden (*Roos* 1956 and *Christensen* 1956) and from U S A (*Mason* and *McLean* 1961) Two mechanisms for the spread of virus with oysters have been postulated one that the virus multiplies in the oysters the second that the oysters only are passive carriers

The second possibility was studied experimentally using poliovirus type 3 as a model strain Oysters were stored alive in aquariums with sea water at 23° C. Samples of oysters and water were drawn after different time intervals and the concentration of virus in samples was titrated in monkey kidney cell cultures

After a short time of exposure less than two hours poliovirus could be found in the oysters in the same concentration as that of the surrounding water No concentration of virus above that level could be demonstrated The virus was located in the soft tissue of the oysters Infective virus was found in the oysters for a considerable long period of time after that the virus in the water had been removed or had become inactivated Suspensions of homogenized oysters had a protective capacity against the inactivation of virus

Holm S & Lycke E Municipal Virological Laboratory and the Department of Bacteriology University of Gothenburg Gothenburg STUDIES ON VACCINIA LS ANTIGEN

The vaccinia LS antigen described by *Craigie* and *Wishart* (1936) is considered to be a protein molecule with two antigenically active sites (*Shedlowsky* and *Smadel* 1942 One of these the L factor is heat labile while the S factor is heat stabile but is degradated by treatment with trypsin The L factor is relatively resistant to trypsinization

In the present studies the LS antigen was analyzed by means of double diffusion in gel technique Four different antigen preparations were made One was a homogenized material of vaccinia virus infected chorioallantoic membranes of chick embryos The second was prepared similarly but was obtained from infected HeLa cell cultures From these crude antigen preparations LS antigens were purified by means of differential centrifugation and isoelectrical precipitation

Antisera were prepared by vaccination of rabbits with purified vaccinia virus material or by subcutaneous injections with the LS antigens When the two crude preparations were tested against rabbit anti vaccinia sera five vaccinia specific immunological systems could be demonstrated One of the five immunological systems found was related to LS anti LS The two types of LS antigen preparations were found to be immunologically identical

After the appropriate treatment for degradation of the L or the S components the LS antigen reacted immunologically as one molecule with two different antigenic active sites

body levels against types 1 and 3 30 per cent of the vaccinees tested had no demonstrable antibodies against the former and 40 per cent against the latter type Antibodies against type 2 were still present in 100 per cent

A group of patients who in 1957 had their primary two shots with inactivated vaccine and their booster with oral live polio vaccine (only type 1) was compared with the above mentioned control study In the live vaccine group antibodies against type 1 could still be demonstrated in 1961 in 100 per cent with mean titres about 1/125

Bengtsson S & Philipson L Institute of Virology University of Uppsala Uppsala
COUNTER CURRENT DISTRIBUTION OF POLIOVIRUS TYPE 1

To be published in Virology 1963

Ohlson Marianne Virus Department Central Bacteriological Laboratory of Stockholm City ANTIGENIC COMPOSITION OF ENTEROVIRUSES IN IMMUNODIFFUSION

A microtechnique with punched holes in agar on microscopic slides was used Native polioantigens prepared from infectious tissue culture fluid showed two precipitating antigens D and C The concentration of C antigen was rather high Different ways of concentration (by cellogel differential centrifugation or polymer phase separation in polyethyleneglycol dextransulphate) yielded the same proportions D C Serial passages with end point diluted inocula did not reduce the C antigen content However, treatment with freon 113 reduced the C antigen more than the D antigen

ECHO 6 apparently had only one precipitating antigen

Immunization with native polio antigens in guinea pigs gave a rise in D and C antibodies Heated antigens (56° C for 30 min) induced C antibodies but to a varying degree also antibodies of D character although the heated antigens had very low infectivity titres (0 7/ml) Homologous D antibodies could be absorbed with native antigen C antibodies with native or heated antigens

Heterotypic reactions between polioviruses occurred after prolonged immunization Polio type 2 hyperimmune sera precipitated with polio type 1 D antigen polio type 1 sera with polio types 2 and 3 antigens (mainly C) neither precipitated with ECHO 6 Individual variation of antibody response was observed

Svart Malmberg Gunvor Department of Virology Central Bacteriological Laboratory of Stockholm City THERMOLABILE SERUM FACTOR INFLUENCING THE NEUTRALIZATION OF ADENOVIRUS

By the technique of *Kjellen et al* (Acta Paediat Stockh 46 164 1957) human sera have been tested for their capacity of inhibiting certain adenovirus types It was demonstrated that a number of sera inhibited adenovirus type 7 Gomen to a greater extent before than after inactivation at 56° C for 30 minutes The addition of fresh guinea pig serum to inactivated samples restored the inhibitory capacity to the level of the unheated sample in several but not all of the sera tested Nor did fresh human serum without effect per se on adenovirus type 7 substitute the factor lost by heat inactivation Decomplementation by means of absorption of the complement on to a precipitate also removed the inhibitory capacity In this case however it was possible—by the addition of guinea pig serum partially and by the addition of

sheep erythrocytes The haemolysin titre was estimated five days later The cell material to be tested was also injected intraperitoneally

It was first found that a homogenate of homologous spleen cells significantly increased the haemolysin response if given a few hours before or simultaneously with the antigen but not when the cell material was injected a few hours later Thymocytes and liver cells were as effective as spleen cells There was no significant difference between autologous and homologous cells in stimulating capacity In the subsequent experiments the antigen and the homologous lymphoid material were given together

Following the observation that whole cells and whole nuclei were not a requisite for the effect, further studies have been devoted to subcellular fractions (obtained by differential centrifugation of desintegrated spleen cells or thymocytes) Most of the activity seemed to reside in the nuclei Microsomes and the 105 000 g supernatant (60 min) had no significant effect Incubation of thymocyte nuclei with DNAase deprived them of the stimulating effect A significant amount of the activity was released from the nuclei during incubation

Laurell Anna Britta Institute of Bacteriology, University of Lund Lund
COMPARISON OF THE AGGLUTINATION OF EA AND EA$_{142}$ CELLS BY RHEUMATOID ARTHRITIC SERA

Rheumatoid arthritis sera were tested on their ability to agglutinate sheep red cells (sensitized by rabbit anti sheep red cell antibodies) EA and such cells complexed with the complement factors C1 C4 and C2 of a human serum pool EA$_{142}$ EA$_{142}$ cells were in contrary to EA cells inagglutinable by rheumatoid arthritic sera Further investigation showed that EA$_1$ blood cells agglutinated to the same extent as EA cells did while all blood cell preparations carrying *the activity of C4* EA$_{14}$, EA$_{41}$ EA$_4$ and EA$_4$ did not agglutinate Haemolytically active C2 on *the sensitized* cells did not seem to inhibit the agglutination

On the other hand a rat anti rabbit γ globulin serum agglutinated EA and EA$_{142}$ *red cells to the same degree* indicating that different antigenic *determinants of the* rabbit amboceptor react with rheumatoid arthritic factor and the anti rabbit γ globulin *antibodies respectively*

Nyström B Central Laboratory for Clinical Bacteriology Karolinska sjukhuset Stockholm PHAGOCYTOSIS IN RHEUMATOID ARTHRITIS

Wilson et al (J Exp Med *106* 777 1957) showed that β haemolysing streptococci engulfed in granulocytes *in peripheral blood* from healthy individuals survived intracellularly for less than 20 minutes

Olhagen has found (to be published) that *it often* is difficult to treat successfully infections with β haemolysing streptococci in patients with rheumatoid arthritis It has therefore seemed of interest to study the intracellular survival time of *strepto*cocci engulfed in *phagocytes* from patients with rheumatoid arthritis A slight modification of the method of *Wilson et al* has been used

In experiments *on 24 healthy* individuals the results were in agreement with those of *Wilson et al*

26 experiments *were made* with granulocytes from patients with rheumatoid arthritis In 8 of them the *streptococci were* still viable after having been engulfed for 30 minutes As a proof of viability was taken the ability of the engulfed streptococci to manifest multiplication after disruption of the granulocyte

Hermodsson S Institute of Virology University of Uppsala Uppsala INHIBITION OF INTERFERON IN DOUBLE INFECTED CELLS

Inoculation of large amounts of Newcastle disease virus (NDV) into calf kidney cultures resulted as a rule in a slight virus multiplication probably due to the presence of an interferon like substance in the inoculated virus fluid The autointerfering activity of NDV however could be inhibited if the cultures prior to the inoculation of NDV was infected with parainfluenza 3 virus (PI-3) An infection with PI 3 could also activate the reproduction of NDV in cultures which had been persistently infected with the latter virus A possible explanation to this phenomenon was that NDV induced to the production of less amounts of interferon in cultures infected with PI 3 than in normal cell cultures and that an infection with PI 3 suppressed the antiviral action of interferon on the multiplication of NDV

Strannegård Ö & Lycke E Municipal Virological Laboratory and the Virological Laboratory of the Department of Bacteriology University of Gothenburg Gothenburg STUDIES ON ANTIGEN ANTIBODY SYSTEMS WITH REFERENCE TO MONONUCLEOSIS INFECTIOSA

Sera from patients with mononucleosis infectiosa contain ox cell haemolysins and agglutinins directed against ox and sheep cells In the present study the ox cell haemolysin (O C H) titres were found to be well correlated to titres obtained in sheep cell agglutination tests (P B tests) The ox cell agglutinins seemed to be identical with the ox cell haemolysins Absorption studies indicated that two or possibly three types of antibodies are reacting in the O C.H and P B tests

Antigenic preparations obtained from heated and mechanically disintegrated ox and sheep erythrocytes gave rise to precipitation lines when allowed to react with sera with high O C H and P B titres The occurrence of precipitins was found to be correlated to the concentrations of ox cell haemolysins and sheep cell agglutinins The diffusion in gel studies demonstrated two antigenic factors in ox erythrocytes One of these factors was related to the only one demonstrated in sheep erythrocytes These findings were supported by the absorption studies

The antibodies precipitating ox cell antigens appeared to be β_{M} globulins

Fjellström K E Department of Clinical Chemistry University Hospital Uppsala ELECTROPHORETIC STUDIES ON HUMAN COMPLEMENT LOCALIZATION OF C FACTORS IN STARCH GEL

To be published in Acta path et microbiol scandinav 1963

Killander J & Hogman C Department of Clinical Chemistry and the Transfusion Service University Hospital Uppsala FRACTIONATION OF HUMAN BLOOD GROUP ANTIBODIES BY GEL FILTRATION

To be published in Scand J Lab Clin Invest 1963

Berglund K Fagraeus Astrid & Skill O Rheumatology Department Karolinska sjukhuset National Bacteriological Laboratory Stockholm and the Institute of Medical Chemistry University of Uppsala Uppsala THE ENHANCING EFFECT OF LYMPHOID CELLS AND CELL FRACTIONS ON ANTIBODY RESPONSE

The test object has been the early phase of the primary haemolysin response in the rat The animals were given one intraperitoneal injection of a small amount of

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Agström B Central Laboratory for Clinical Bacteriology Karolinska sjukhuset Stockholm

PHAGOCYTOSIS IN RHEUMATOID ARTHRITIS

Wilson *et al* (J Exp Med *106* 777 1957) showed that β haemolysing streptococci engulfed in granulocytes in peripheral blood from healthy individuals survived intracellularly for less than 20 minutes

Ohlsgren has found (to be published) that it often is difficult to treat successfully infections with β haemolysing streptococci in patients with rheumatoid arthritis It has therefore seemed of interest to study the intracellular survival time of streptococci engulfed in phagocytes from patients with rheumatoid arthritis A slight modification of the method of *Wilson et al* has been used

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Berglund K Lagraeus Astrid & Sköld O Rheumatology Department Karolinska sjukhuset National Bacteriological Laboratory Stockholm and the Institute of Medical Chemistry University of Uppsala Uppsala THE ENHANCING EFFECT OF LYMPHOID CELLS AND CELL FRACTIONS ON ANTIBODY RESPONSE

The test object has been the early phase of the primary haemolysin response in the rat The animals were given one intraperitoneal injection of a small amount of

Lindberg A & Kallings L O National Bacteriological Laboratory Stockholm
ROLE OF LYSOGENICITY IN 0-1 RESISTANT SALMONELLA BACTERIA

The bacteriophage 0 1 acting on most Salmonella bacteria separates the Salmonella strains into two groups strains sensitive to low phage concentrations and supporting the propagation of new particles and strains only influenced by high concentrations and unable to produce phages (*Kallings* 1961) The purpose of this paper is to study the cause of the different sensitivity to the phage As the resistance was assumed to be due to latent phages blocking the synthesis of 0-1 phages experiments are reported concerning the isolation of latent phages and attempts to transform sensitive strains to resistant ones by means of these phages

Twenty Salmonella strains were chosen and tested for 0-1 phage activity One of the two resistant strains was induced by exposure to ultraviolet radiation the supernate was then tested against the other strains by spot technique A temperate phage (105) was isolated and found able to transform the sensitive S typhi murium strains to resistant ones The 0-1 resistant cultures were induced to prove that they were lysogenized by phage 105 The supernates exhibited the same lytic spectrum as phage 105 Their activity was neutralized by hyperimmune serum against phage 105

Phages with the same ability to change the sensitivity to phage 0-1 were isolated from other cultures belonging to different salmonella groups

Danielsson D & Laurell G Institute of Bacteriology University of Uppsala Uppsala RAPID DETECTION OF SMALL NUMBERS OF BACTERIA IN WATER BY MEANS OF FLUORESCENT ANTIBODIES

Fluorescent antibody technique combined with membrane filter technique has been used to study bacterial contamination of water known concentrations of the test bacteria enteropathogenic Esch coli were added to fixed volumes of water As a rule 1 litre was filtered through a membrane filter (Millipore HAWG 47 mμ) At bacterial densities of 500–1000 bacteria/litre identification was accomplished in two hours At lower concentrations the membrane filters were incubated for varying periods in broth and subsequently centrifuged to concentrate the bacteria By this technique a bacterial concentration of 50 bact./litre could be detected in 5 6 hours 15 20 bact./litre in 8-10 hours and 2 5 bact./litre in 12–16 hours respectively The technique was also quantitative at different ratios (0 2 100) between concentration of contaminating bacteria and test bacteria By conventional bacteriological and serological tests diagnosis could be made after 48 hours at the earliest and at a high ratio of contaminating bacteria to test bacteria the latter could often not be isolated

Promising attempts have been made to detect bacteria directly on nonfluorescent membrane filters (Millipore HABG) by the use of fluorescent antibodies This method offers a chance of further reducing the time

In a 6 months field study samples of the water of a river in central Sweden (the Fyris were examined as to the occurrence of enteropathogenic Esch coli By the use of fluorescent antibodies 10 different strains of these bacteria were detected 6 of these were also isolated by conventional tests

The experimental conditions are too uncertain to allow any positive conclusions to be drawn. Further experiments are planned with a technique that allows quantitative estimation of engulfment and cytopepsis.

Hederstedt B. National Bacteriological Laboratory, Stockholm, Sweden.
TREPONEMA PALLIDUM IMMOBILIZATION IN NORMAL SERUM

Treponema pallidum immobilization is demonstrable in unheated normal serum from man and certain animals.

Aliquots of unheated serum and treponemes, suspended in a modified (e.g. reducing SH compounds replaced by aq. dest.) Nelson medium were mixed and incubated at 35° C in CO_2-N_2 atmosphere for 90 minutes.

The immobilizing activity was found in the majority of healthy persons studied though in low dilutions only ($\leqq$ 1:16). This serum activity occurred in rat, sheep and ape, but not in guinea pig, rabbit or hamster.

The immobilizing serum activity disappeared after heat-inactivation or decomplementation with specific precipitate. No activity was found in R_1, R_2, R_3 or R_4 serum. Zymosan treatment at 17° C did not abolish the immobilizing activity. This activity was lost after bentonite treatment and was restored by egg white lysozym. There was no quantitative correlation between immobilizing effect and complement or lysozym activity in different sera.

The non-specific immobilizing activity did not seem to affect the result of the specific TPI test, probably due to the use in this test of heated human serum and an inhibiting effect of guinea pig and rabbit sera on the non-specific immobilizing activity.

Lindbom G. Institute of Bacteriology, University of Uppsala, Uppsala. **THE GENESIS AND INTRAMURAL SPREAD OF A STAPHYLOCOCCAL EPIDEMIC**

During 1961 the University Hospital in Uppsala was troubled by a series of infections where Staph. aureus phage type 75/77 was isolated. This strain had been uncommon in the preceding years.

The first cases were noticed in March, when patients undergoing thoracic surgery developed postoperative wound indfections caused by highly antibiotic resistant staphylococci. After 3 months 8 patients, 2/3 of whom had been tracheotomized, had been infected. Circumstantial evidence seemed to indicate the postoperative unit as major source of infection.

Retrospective analysis of epidemiological data from the whole hospital strongly suggested that the 75/77 strains were brought to Uppsala from a regional hospital by a few patients already infected and sent here for specialized treatment. Portals of entry were the departments of orthopedics, plastic and thoracic surgery. The strain was dispersed to other parts of the hospital by patients transferred for diagnostic and/or therapeutic purposes. In June all major specialities were affected by the epidemic. Finally the strain was spread to local hospitals by patients returning for further treatment.

All in all 47 patients infected by this strain could be traced; it was an important factor in the death of 8 patients.

This epidemic demonstrated both the spread of staphylococcal disease from one hospital to another and within one hospital.

FROM THE DEPARTMENT OF PHYSIOLOGY AND THE DEPARTMENT OF PATHOLOGY II UNIVERSITY OF GÖTEBORG GÖTEBORG SWEDEN

ACTION OF OVARIAN HORMONES ON THE MAMMARY GLANDS OF ALLOXAN DIABETIC RATS

By

K. AHRÉN and L. ANGERVALL

Received 13 xi 62

Ovarian hormones promote growth and development of the mammary glands in animals with intact pituitary gland. Thus, in most species administration of oestrogens alone induces ductal growth in the mammary glands, and administration of oestrogens combined with progesterone stimulates both ductal growth and alveolar development. It has been known for a long time, however, that ovarian hormones fail to stimulate the mammary glands in hypophysectomized animals, and it has also been shown that known hypophysial factors combined with ovarian hormones can promote mammary gland development in such animals (for lit. see *Folley* 1952, *Lyons* 1958, *Jacobsohn* 1961).

Observations published in recent years suggest that also insulin is capable of influencing growth processes within the mammary glands. *Ahrén & Jacobsohn* (1956), for example, reported that the mammary glands showed development in hypophysectomized rats treated with ovarian hormones and insulin but remained undeveloped in similar rats given ovarian hormones alone. It has not been established, however, whether the ability of insulin to affect growth processes within the mammary glands, as observed by *Ahrén & Jacobsohn*, is due to a direct action of this hormone on the mammary parenchymal cells or only to its influence upon the intermediate metabolism. In addition, it is unknown whether the presence of insulin is an absolute requirement for the growth processes of the mammary glands to take place *in vivo*. Many recent observations suggest that such is the case in tissue cultures (*Elias* 1959, *Prop* 1960, *Rivera & Bern* 1961).

The present investigation was designed to study the effect of ovarian hormones on the mammary glands of castrated, alloxan diabetic rats, particularly to ascertain if ovarian hormones are capable of stimulating growth processes within the mammary glands of markedly insulin deficient rats.

We a ... assista ... nical ... were k ... tudy ... defray ... were

Kallings L O Bucht H Wehle B & Örsten P National Bacteriological Laboratory and the IVth Medical Clinic (The Kidney Clinic) St Eriks Hospital Stockholm
TREATMENT OF CHRONIC PYELONEPHRITIS WITH AMPICILLIN

Infections due to *Proteus mirabilis Escherichia coli* and *Aerobacter aerogenes* in 18 cases of chronic pyelonephritis were treated with 0 75 to 4 0 g ampicillin orally daily during periods varying from some weeks to a year Most cases were previously found to resist treatment with other drugs

The sensitivity of the bacteria to ampicillin was tested and the ampicillin concentration in serum and urine determined The treatment was followed clinically with special reference to the kidney function and by assay of the bacterial concentration in the urine The influence on the intestinal flora was examined by cultivation and determination of stercobilin and bilirubin in faeces and of pyruvic acid in serum

At the level of 10^5–10^6 cells/ml the MIC in broth varied with the different bacterial strains from 3 to 12 500 µg/ml

Clinical improvement occurred in most cases Sterile urine was obtained in 6 cases and the bacterial counts were markedly reduced in 4 cases Failures were obtained in patients infected with aerogenes or with the most resistant proteus bacteria and in those with renal calculi The normal intestinal flora was temporarily depressed in a few cases No complications occurred

Tunevall G & Frisk R Central Bacteriological Laboratory of Stockholm City
STUDIES ON PENICILLINS OF THE PHENOXYMETHANE SERIES

The compounds studied are phenoxy methyl aethyl propyl and isopropyl butyl and isobutyl amyl and hexylpenicillin For comparison benzyl α amino benzyl and 2 6 dimethoxyphenylpenicillin were included

The general *in vitro* activity against sensitive organisms decreased with increasing length of the side chain though at most by two steps in a twofold dilution series Against penicillinase producing staphylococci an increase in activity from benzyl to phenoxyhexylpenicillin of the same magnitude was found Iso forms were slightly less active than normal ones but more stable against penicillinase

In triple crossover experiments in groups of ten patients the serum concentrations after single oral doses of 0 3 g were followed Higher and more lasting concentrations were found the longer was the side chain After 1 hour phenoxyhexylpenicillin gave 5 times the concentration of phenoxymethylpenicillin after 6 hours as much as 15 times

Winge Hedén Kerstin Central Laboratory of Clinical Bacteriology Karolinska sjukhuset Stockholm
STERILIZATION BY ETHYLENE OXIDE WITHOUT SPECIAL EQUIPMENT

To be published in Acta path et microbiol scandinav 1963

Marked glucosuria (lowest 1 5 and highest 2 9 g per 24 hours) and inhibited body growth were noted in 6 of the 8 rats in the alloxan group The 2 remaining rats in this group did not show glucosuria, and hence were considered to have no manifest diabetes As appears from the tables and from Figs 3 and 4, these 2 rats responded in the same way as the controls in all other respects as well No change in the severity of diabetes, as reflected by the degree of glucosuria, could be demonstrated during the fortnight of ovarian hormone treatment

TABLE 1

Weights of Adrenals and Thymus in Control and Alloxan Diabetic Rats

	No of rat	Body weight (g) Before injections of Alloxan	Body weight (g) Before injections of Ovarian hormones	Body weight (g) At autopsy	Adrenal weight (mg)	Thymus weight (mg)
A Control rats	C1	110	170	183	53 8	160
	C2	118	190			
	C3	86	140	154	40 4	104
	C4	105	170	183	53 9	198
	C5	89	129	147	42 9	215
	C6	93	130	145	58 5	206
	C7	83	140	155	43 5	289
	C8	85	125	137	31 5	145
	C9	77	125	148	38 7	218
Mean weight ± standard error of the mean		91±4	141±7	157±7	45 4±3 2	192±20
B Rats injected with alloxan Manifest diabetes	Ax2	105	139	156	48 5	113
	Ax3	87	95	119	44 5	122
	Ax4	84	100	118	48 9	81
	Ax5	96	122	129	60 3	74
	Ax6	71	78	83	52 4	22
	Ax8	90	110	120	49 5	68
Mean weight ± standard error of the mean		89±5	107±9	121±10	50 7±2 2	80±15
C Rats injected with alloxan No manifest diabetes	Ax1	119	182	191	51 7	149
	Ax7	117	149	145	47 6	178

At autopsy the diabetic rats had a body weight significantly lower than the controls ($P < 0.01$) The absolute weight of the adrenals in the former was higher but not significantly so ($P = 0.2$) In relation to body weight the diabetic rats apparently had a higher adrenal weight than the controls The lower thymus weight in the diabetic rats ($P < 0.01$) seems only partly explained by the body weight difference

MATERIAL AND METHODS

The experiments were made on a group of 17 male rats of a Wistar strain maintained for many years at the *Department of Pathology Born 1 March 1961* the rats were castrated between the 19th and 28th day of age, when they weighed 35 to 40 g From 21 to 31 days after being castrated 8 of the rats received a subcutaneous injection of 0 15 mg alloxan per g body weight The remaining 9 rats were used as controls *When 21 days had elapsed after the alloxan injection the 3rd right thoracic* mammary gland was extirpated for whole mount preparation both from experimental rats and controls During the next 14 days, all the rats were given daily intramuscular injections of 10 µg oestrone (100 µg/ml in arachis oil) and 4 mg *progesterone* ("*Progestin*" *brand Pharmacia 25 mg per ml)* The day after the last injection the rats were exsanguinated under ether, the adrenals and thymus being excised and weighed The 3rd left thoracic and the left abdominal mammary glands were excised for preparation of whole mounts These specimens were prepared and stained with gallocyanine chromalum as described previously (*Ahrén & Etienne* 1957, *Ahrén* 1959 b) For histological examination the corresponding right abdominal mammary gland was excised fixed in Bouin's solution, embedded in paraffin and cut into 5 µ sections which were stained with haematoxylin eosine and according to Weigert van Gieson

All the rats were given tap water and a special, commercial rat bread *ad libitum* throughout the experiment All were weighed at regular intervals of about a week The degree of glucosuria (in g per 24 hours) was estimated by Benedict s quantitative method During periods when the urinary glucose output was estimated the rats were transferred to separated cages adapted especially for urine collection some contamination of the urine by food was unavoidable

The whole mount mammary gland preparations were examined microscopically in order to assess the type and degree of glandular development In so doing we adopted a scoring system slightly modified in accordance with the below definitions introduced by *Cowie & Folley* (1947) and used over a period of years by one of us (*e g Ahrén* 1959 b)

Duct System Development

- \+ Restricted duct system showing little branching
- ++ Moderately extensive duct system showing moderate branching
- +++ Widespread duct system showing profuse branching

Club-Shaped End Buds

- \+ Few end buds present
- ++ Numerous end buds irregularly disposed around the periphery
- +++ Abundant end buds regularly disposed around the periphery

Alveolar Development

- \+ Few alveoli present
- ++ Moderate alveolar development, evenly distributed
- +++ Ducts covered with alveoli

In the column for alveolar development in the table the score '(+)' will also be found This score was applied to glands exhibiting a few indeterminate formations which however might have been alveoli For as shown by *Richardson* (1947) to distinguish clusters of small ducts from true alveoli may be very difficult or even impossible

RESULTS

Table 1 gives body weights at different times during the experimental period and weights of the adrenals and thymus at autopsy Table 2 gives a synopsis of mammary gland development before and after treatment with ovarian hormones Figs 1–10 illustrate typical whole mount preparations and paraffin sections of mammary glands from alloxan diabetic rats and controls

PLATE I









TABLE 2

Synopsis of Mammary Gland Development before and after Treatment with Ovarian Hormones in Control and Alloxan Diabetic Rats

	No of rat	Mammary glands					
		Duct system		End buds		Alveoli	
		Before ovarian hormones	After ovarian hormones	Before ovarian hormones	After ovarian hormones	Before ovarian hormones	After ovarian hormones
A *Control rats*	C1	++	+++	+	++	0	+++
	C2	++	+++	0	+++	(+)	+++
	C3	++	+++	+	++	0	+++
	C4	++	+++	++	++	0	+++
	C5	++	+++	0	+	0	++
	C6	++	+++	++	++	0	+++
	C7	++	+++	0	++	0	++
	C9	++	+++	+	++	0	++
	C8	++	+++	+	++	0	++
B *Rats injected with alloxan Manifest diabetes*	Ax2	+	++	0	+++	(+)	+
	Ax3	+	++	0	++	(+)	+
	Ax4	+	++	0	+++	(+)	+
	Ax5	+	++	0	++	(+)	+
	Ax6	+	++	0	++	0	+
	Ax8	+	++	0	++	(+)	+
C *Rats injected with alloxan No manifest diabetes*	Ax7	++	+++	++	+	0	+++
	Ax1	++	+++	+	++	(+)	+++

Comments to Plates I III

Figs 1 8 show whole mount preparations of mammary glands stained with gallocyanin chromalum and photographed at the same magnification (*see Plate II*) Figs 9 and 10 show microphotographs of 5 μ thick sections through mammary glands stained with Weigert van Gieson The microphotographs were taken at the same magnification (× 110)

Plate I

Fig 1 (Table 1 C 6) Castrated male rat Third right thoracic gland extirpated before the injections of ovarian hormones —Small gland No alveoli Many small end buds

Fig 2 Same rat as in Fig 1 Third left thoracic gland after 14 days of treatment with 10 μg oestrone and 4 mg progesterone daily —This gland covered a very large area and the photograph shows approximately one fourth of the gland —The duct system is markedly developed and there is an extensive lobule alveolar development

Fig 3 (Table 1 Ax 7) Castrated male rat injected with alloxan but *without manifest diabetes* Third right thoracic gland extirpated before the injections of ovarian hormones —Small gland No alveoli Many small end buds

Fig 4 Same rat as in Fig 3 Third left thoracic gland after 14 days of treatment with 10 μg oestrone and 4 mg progesterone daily The photograph shows approximately one fourth of the gland —Marked development of the duct system and extensive lobule alveolar development

PLATE I





TABLE 2

Synopsis of Mammary Gland Development before and after Treatment with Ovarian Hormones in Control and Alloxan Diabetic Rats

	No of rat	Mammary glands					
		Duct system		End buds		Alveoli	
		Before ovarian hormones	After ovarian hormones	Before ovarian hormones	After ovarian hormones	Before ovarian hormones	After ovarian hormones
A Control rats	C1	++	+++	+	++	0	+++
	C2	++	+++	0	+++	(+)	+++
	C3	++	+++	+	++	0	+++
	C4	++	+++	++	++	0	+++
	C5	++	+++	0	+	0	++
	C6	++	+++	++	++	0	+++
	C7	++	+++	0	++	0	++
	C9	++	+++	+	++	0	++
	C8	++	+++	+	++	0	++
B Rats *injected with* alloxan Manifest diabetes	Ax2	+	++	0	+++	(+)	+
	Ax3	+	++	0	++	(+)	+
	Ax4	+	++	0	+++	(+)	+
	Ax5	+	++	0	++	(+)	+
	Ax6	+	++	0	++	0	+
	Ax8	+	++	0	++	(+)	+
C Rats injected with alloxan No manifest diabetes	Ax7	++	+++	++	+	0	+++
	Ax1	++	+++	+	++	(+)	+++

Comments to Plates I-III

Figs 1-8 show whole mount preparations of mammary glands stained with gallocyanin chromalum and photographed at the same magnification (see Plate II) Figs 9 and 10 show microphotographs of 5 μ thick sections through mammary glands stained with Weigert van Gieson The microphotographs were taken at the same magnification (× 110)

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Fig 4 Same rat as in Fig 3 Third left thoracic gland after 14 days of treatment with 10 μg oestrone and 4 mg progesterone daily The photograph shows approximately one fourth of the gland —Marked development of the duct system and extensive lobule alveolar development

Mammary Gland Development

A Before Administration of Ovarian Hormones

All of the diabetic rats had a duct system which was less developed than that in the controls (Table 2 and Figs 1, 3, 5 and 7) In addition most of the controls exhibited club shaped end buds (Figs 1 and 3), but none of the diabetic rats had such end buds (Table 2, Figs 5 and 7) In the controls alveoli were absent in the mammary glands The mammary glands in the diabetic rats lacked distinct alveoli but most of these animals presented some small formations which could have been either clusters of alveoli or groups of small ducts

B After Administration of Ovarian Hormones

Table 2 and Figs 2, 4, 6 and 8 reveal that during the period of hormone treatment the duct system had developed markedly both in diabetic rats and controls Nevertheless, the controls still displayed better duct system development and greater glandular area than the diabetic rats All mammary glands from both groups exhibited numerous club-shaped end buds (Figs 2, 4, 6 and 8), the degree of end-bud development in the diabetic group being at least as high as in the control group

All mammary glands from the controls showed pronounced alveolar development and in the majority of these glands the entire duct system was covered with groups of alveoli (Figs 2 and 4) The mammary glands from the diabetic rats showed a clear but slight alveolar development (Figs 6 and 8) which in all cases was less marked than in the controls

Mammary gland paraffin sections from the controls exhibited a multiplicity of large groups of small alveoli in which the lumen, if present, was small (Figs 9) In the glands from the diabetic rats the alveolar groups were much fewer (Fig 10) The alveolar epithelium in the latter group was somewhat higher than that in the controls, and,

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Plate II

Fig 5 (Table 7 Ax 2) Castrated male rat injected with alloxan and with manifest diabetes Third right thoracic gland extirpated before the injections of ovarian hormones —Very restricted duct system No end buds Eventually a few small groups of alveoli

Fig 6 Same rat as in Fig 5 Third left thoracic gland after 14 days of treatment with 10 μg oestrone and 4 mg progesterone daily —Marked growth of the duct system Large club shaped end buds Very slight alveolar development in the central part of the gland

Fig 7 (Table 1 Ax 4) Castrated male rat injected with alloxan and with manifest diabetes Third right thoracic gland extirpated before the injections of ovarian hormones —Very restricted duct system No end buds Eventually a few small groups of alveoli in one part of the gland

Fig 8 Same rat as in Fig 7 Third left thoracic gland after 14 days of treatment with 10 μg oestrone and 4 mg progesterone daily —Marked growth of the duct system Many large end buds Slight alveolar development in the central part of the gland

PLATE II



5



6



7



5 mm



in addition, the alveolar lumen in the diabetic rats was larger than in the controls

DISCUSSION

The present investigation has demonstrated that ovarian hormones can stimulate an extensive ductal growth and a slight but clear alveolar development in the mammary glands of alloxan diabetic rats This result shows that a normal insulin secretion from the islet β cells is not a prerequisite for the stimulation of mammary gland growth by ovarian hormones in the rat This result, however, does not prove that the ovarian hormones *in vivo* can stimulate growth processes within the mammary glands in complete absence of insulin, because an alloxan diabetic rat cannot be considered as an "insulin free" animal

After administration of ovarian hormones the duct system of the mammary glands in the two groups of rats showed approximately the same growth increment although the duct system was less developed in the diabetic rats both before and after administration of the ovarian hormones Many observations (*e g Lyons et al* 1955, 1958 *Ahren* 1959 a) indicate that the presence of somatotrophic hormone is required if the ovarian hormones are to be capable of stimulating a more marked duct growth in the mammary glands of the rat Hence the ability of the pituitary to elaborate somatotrophic hormone does not seem to be significantly impaired in the alloxan diabetic rat and, judging by the well developed duct system in their mammary glands it could very possibly be normal or even increased Published reports on the production of somatotrophic hormone in alloxan diabetic rats are conflicting Thus it has been reported that in such rats the somatotrophic hormone activity of plasma is reduced (*Lawrence et al* 1958), whilst that of the pituitary is increased (*Solomon* 1958)

The degree of alveolar development after steroid treatment was less pronounced in the alloxan diabetic rats than in the controls This might indicate that ovarian hormones are unable to induce normal alveolar growth in the mammary glands unless insulin is present in sufficient amounts implying that insulin deficiency was directly responsible for the inhibited alveolar development in the alloxan diabetic rats Considering that ovarian hormones are unable to promote alveolar growth in the mammary glands of the rat unless prolactin is present (*e g Lyons et al* 1955 1958 *Ahren* 1959) another possibility to explain the poor alveolar development in the alloxan diabetic rats is that inadequate prolactin production rather than insulin deficiency was the direct cause of the alveolar underdevelopment Many observations suggest that the secretion of FSH and LH is impaired in diabetic animals (*e g Lawrence & Contopoulos* 1960 a, b *Hunt & Baily* 1961) Whether such animals also have reduced prolactin secretion has apparently not been studied but it seems far from improbable that such is the case

Another point worth considering is the fact that the production of

PLATE III



Fig 9 (Table 1 C 7) Castrated male rat—Right abdominal gland after 14 days of treatment with 10 µg oestrone and 4 mg progesterone daily—Many large groups of alveoli

Fig 10 (Table 1 Ax 2) Castrated male rat injected with alloxan and with manifest diabetes Right abdominal gland after 14 days of treatment with 10 µg oestrone and 4 mg progesterone daily – Very few but somewhat more open alveoli than in the glands from the control rats(compare Fig 9)

in addition, the alveolar lumen in the diabetic rats was larger than in the controls

DISCUSSION

The present investigation has demonstrated that ovarian hormones can stimulate an extensive ductal growth and a slight but clear alveolar development in the mammary glands of alloxan diabetic rats This result shows that a normal insulin secretion from the islet β cells is not a prerequisite for the stimulation of mammary gland growth by ovarian hormones in the rat This result, however does not prove that the ovarian hormones *in vivo* can stimulate growth processes within the mammary glands in complete absence of insulin because an alloxan diabetic rat cannot be considered as an insulin free animal

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Another point worth considering is the fact that the production of

corticosteroid apparently is enhanced in alloxan diabetic rats (cf *Dury* 1953, *Weller & Bettge* 1954, *Kalant* 1955, *Angervall* 1959) In our experiments adrenal hyperplasia combined with thymus atrophy are morphological signs of increased secretion of corticosteroids It has been shown that some corticosteroids under certain experimental conditions can stimulate growth processes within the mammary glands (*Selye* 1954, *Johnson & Meites* 1955) Other studies indicate, however, that the same corticosteroids under other experimental conditions can inhibit such growth processes (*Flux* 1954, *Ahren & Jacobsohn* 1957) Therefore, it is impossible on the basis of the present experiments, to draw any conclusions as to whether the increased production of corticosteroids may have influenced significantly the mammary glands of the alloxan diabetic rats

SUMMARY

Mammary gland development in castrated, alloxan diabetic rats has been assessed before and after treatment with ovarian hormones (10 μg oestrone and 4 mg progesterone daily for 14 days) Before such treatment the mammary glands of the alloxan diabetic rats were less developed than those of corresponding controls The ovarian hormones stimulated in the alloxan diabetic rats a marked growth of the mammary duct system and a slight but clear alveolar development These findings are discussed in the light of our present knowledge about the production in the alloxan diabetic rats of such hormones as have been shown to exert a significant growth promoting action on the mammary glands of the rat

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MAMMARY GLAND DEVELOPMENT IN NEWBORN OFFSPRING OF ALLOXAN DIABETIC RATS

By

K. AHRÉN and L. ANGERVALL

Received 13 xi 62

In the pregnant rat diabetes seems to imply hormonal disturbances which can both enhance and inhibit foetal growth. It is imperfectly understood why diabetic rats can give birth to large offspring. The hypothesis has been advanced that maternal pituitary growth hormone (STH) is responsible for the enhanced growth. Increased production of growth promoting foetal insulin is another debated possibility. Moreover, the foetuses of non-insulinized alloxan diabetic rats are probably influenced by an increased production of maternal corticosteroids which might well inhibit foetal growth (*Angervall* 1959).

Until puberty the development of the mammary glands in the rat parallels body growth (*Astwood et al.* 1937, *Cowie* 1949). When the production of ovarian hormones in appreciable amounts begins during puberty, these hormones induce growth and differentiation of the mammary glands. However, the action of ovarian hormones on the mammary glands is influenced by STH and prolactin (*e.g. Folley* 1952). Under certain conditions growth processes in the mammary glands of the rat can also be affected by insulin (*Ahrén & Jacobsohn* 1956, *Ahrén* 1959). In addition some corticosteroids have been reported to be capable of stimulating the growth and development of the mammary glands (*Selye* 1954, *Johnson & Meites* 1955).

Accordingly, since the mammary gland is an effector organ for several of those hormones which appear to abnormally influence foetuses of alloxan diabetic rats, it should be interesting to study the mammary glands in these rats.

MATERIALS AND METHODS

The experiments were done on male and female offspring of primiparous albino rats (Wistar strain)—25 young from 10 alloxan diabetic rats and 21 young from 6 normal rats. Diabetes was induced by a single subcutaneous injection of 0.15 mg per g rat of 5 per cent alloxan solution given on the 10th, 11th or 12th day of pregnancy. The rats were given a special rat bread and tap water *ad libitum*. Benedict's quantitative method was employed for estimating the degree of glucosuria. The alloxan diabetic rats had a urinary sugar output ranging from 2 to 4 g per 24

hours. As soon as the offspring had been born spontaneously they were weighed and decapitated. The weight of the 25 newborns in the diabetic group ranged between 3.76 and 6.97 g and that of the 21 newborns in the control group between 4.32 and 5.71 g.

The skin with the mammary glands of one half of the body was removed, stretched on tracing paper and fixed in Carnoy's solution for 1? to 24 hours. After dehydration with alcohol (1 hour each of absolute, 96 per cent, 80 per cent and 70 per cent alcohol) the 3rd thoracic mammary gland was excised under a dissection microscope, stained in gallocyanin-chromalum for 12 to 24 hours and mounted as a whole specimen on a glass slide.

The thoracic mammary glands of the other half of the body were rid of excess skin, fixed in 10 per cent formalin, embedded in paraffin and sliced into 5 μ sections which were stained with haematoxylin-eosine.

RESULTS

Whole specimens. The 25 mammary glands from the offspring in the diabetic group showed primary, secondary and tertiary ducts radiating from the mammillary region in a plane parallel to the surface of the skin (Figs. 1 and 2). In 22 glands short side buds projected from these main ducts which generally were terminated with distinct, club shaped end buds. In 3 young from the same litter side-buds were lacking, although these too had distinct end buds. The mammary glands from the controls exhibited the same features in principle (Figs. 3 and 4). Thus the mammary glands from the diabetic and control groups exhibited no significant qualitative or quantitative difference.

Paraffin sections. The main glandular ducts in this category were consistently open ended. They were demarcated by a fairly tall layer of cylindrical epithelium. Here and there outside this layer there was a layer of lower epithelial cells of various sizes. All epithelial cells exhibited a spheroid or ellipsoid nucleus which was moderately rich in chromatin and possessed a distinct nucleolus. Cells in mitosis were plenty. Corresponding to the side buds observed in the whole specimens there were small compact clumps of epithelium. The offspring in the diabetic and control groups exhibited no manifest differences.

DISCUSSION

In the present investigation no manifest differences in mammary gland development were found between offspring of non insulinized alloxan diabetic rats and control rats. The appearance of the glands showed good agreement with previously published data regarding the appearance of the mammary gland in newborn normal albino rats (*Myers* 1916, 1919; *Astwood et al.* 1937).

Some observations suggest that—unlike the case in adult rats—the foetal and early extrauterine development of the mammary glands is unaffected by ovarian hormones (*Astwood et al.* 1937). It has not been demonstrated whether foetal mammary gland development can be disturbed by other hormones which are capable of interfering with growth processes in mammary glands of the adult rat, such as STH, prolactin

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By

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Received 13 xi 6?

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Figs 1–4

show whole mount preparations of mammary glands stained with gallocyanin chromalum and photographed at the same magnification

Fig 1 Third thoracic mammary gland from a newborn offspring of an alloxan diabetic rat Distinct end buds are seen

Fig 2 Third thoracic mammary gland from a newborn offspring of an alloxan diabetic rat Distinct end buds Distinct but small side buds

Fig 3 Third thoracic mammary gland from a newborn offspring of a control rat Distinct end buds are seen

Fig 4 Third thoracic mammary gland from a newborn offspring of a control rat Distinct end buds Distinct but small side buds

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insulin and certain corticosteroids The outcome of the present investigation suggests that the hormonal disturbance existing in offspring of alloxan diabetic rats is not reflected in the development of their mammary glands The reason may be either that foetal mammary gland development is independent of at least some of those factors which regulate foetal growth of the body, or that factors promoting and factors inhibiting foetal mammary gland development are equally abundant in offspring of alloxan diabetic rats It is interesting to note in this connexion that certain corticosteroids (11-oxysteroids), which seem to be present in excess in foetuses of non-insulinized alloxan diabetic rats, may exert an inhibitory action on the development of the mammary glands (*Flax* 1954)

SUMMARY

The authors have studied the development of the mammary glands in newborn offspring of alloxan diabetic albino rats No manifest differences from control offspring were noted The observations are discussed in the light of our present knowledge regarding the hormonal imbalance in alloxan diabetic pregnant rats and their offspring

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THE INSTITUTE OF PATHOLOGY AND THE INSTITUTE OF BACTERIOLOGY
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NEOPLASMS IN GUINEA PIGS INDUCED BY AN AGENT IN ROUS CHICKEN SARCOMA[1]

By

CARL GUSTAF AHLSTRÖM, SVEN BERGMAN
and BENGT EHRENBERG

Received 13 xii 62

It has recently been reported that certain strains of Rous sarcoma virus are capable of producing sarcomas and haemorrhagic cysts in rats (2 5 7 11, 13 15–20), sarcomas in mice (2, 6) and hamsters (3), and fibrosarcoma like tumours and lymphogenous cysts in rabbits (2, 6, 19 20) This paper describes the development of tumours in guinea pigs inoculated with Rous sarcoma material (Schmidt- Ruppin strain) A description will also be given of the growth of the guinea pig tumours *in vitro* as well as of the results of inoculation of guinea pig fibroblast cultures with Rous virus

MATERIAL AND METHODS

Virus strains Two strains of the Rous sarcoma virus were used One strain *the Schmidt Ruppin strain* was obtained from Dr *H Schmidt Ruppin* in Frankfurt A M/Hoechst and is capable of inciting neoplasia in rats mice hamsters and rabbits (2 7 11) The other strain the *Mill Hill strain* was obtained from Sir *Christopher Andrewes* National Institute of Medical Research London Rats mice hamsters and rabbits are not susceptible to this strain (4) Inoculation of either virus strain into chickens will within 7–10 days induce a neoplasm with the usual gross and m
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Animals

The guinea pigs (Cavia Cobaya) were purchased from a commercial dealer and inoculated intramuscularly in the right hind leg within the first week of birth (weight 70–240 grams) Adult guinea pigs weighing 375–700 grams were also inoculated The guinea pigs were fed standard pellets and fresh greens They were examined every third day for the first month after inoculation then once a week or once a fortnight

Inoculation

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[1] This work was supported by research grants 61 5 and 62 6 from the *Swedish Cancer Society* and by research grant CA 06415 01 from the *National Institutes of Health Public Health Service*

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Ruppin strain) Of the 10 guinea pigs inoculated with repeated doses, all but one developed tumours In only one of the adult guinea pigs did a tumour appear

Of the 43 guinea pigs inoculated with cell free extract of the chicken sarcoma (Schmidt-Ruppin strain), 36 developed tumours Positive results were obtained in all the 8 guinea pigs which had been inoculated repeatedly but negative in 2 of the 8 which had received the same total amount of extract in a single dose 6 of the 8 guinea pigs that had received intraperitoneal injections of the extract developed tumours in the abdominal wall at the site of the needle puncture No tumours were seen in the abdominal cavity

When tumours developed, they invariably *did so at the site of inoculation* They usually appeared in the form of a nodule or sometimes as a group of nodules 23–24 days after the inoculation but occasionally within 17 days In some animals, however, the latent period covered up to 2–3 months

In the initial stage the tumours increased only slowly in size They were rounded and sometimes irregular, they felt firm and fibrous, and they were usually adherent to the surrounding muscles (Fig 1) The further development varied After a period of slow growth the tumour often suddenly increased rapidly in size, expanded the thigh and bulged into the pelvis with consequent severe disability of the animal At the same time the tumour became softer In other animals the tumour became adherent to the overlying skin, which turned bluish-violet and finally ulcerated Necrotic material was discharged from the floor of the ulcers (Fig 1) In some cases the tumour then regressed, in some the host died from a secondary infection In others the tumour gradually became harder and smaller and left behind a fibrous scar, often with consequent permanent contracture of the hind leg The size of the tumour ranged from 4 × 4 × 4 5 cm to 0 5 × 0 5 × 0 5 cm

The cut surface was usually greyish-white with irregular areas of yellowish dry necrosis Rapidly growing tumours had a moist white medullary cut surface and often showed central haemorrhages (Fig 2) The tumours were fairly well outlined against the surrounding tissue In some cases metastases were seen in the lymph nodes adjacent to the tumours, in others the inguinal and retroperitoneal lymph nodes showed only an unspecific inflammatory reaction The lungs in 4 guinea pigs were the site of numerous, hard, rounded, greyish-white tumour-nodules often surrounded *by a narrow haemorrhagic zone* (Fig 3) No metastases were seen in the liver, kidneys or spleen

Histologically the tumour usually had the character of a spindle-cell sarcoma built up of densely crowded elongated cells, arranged in an irregular network of bundles (Fig 4) Often two types of cells could be distinguished (Fig 5) One type had a narrow nucleus fairly rich in chromatin the cells were oblong and the cytoplasm scanty The other type had a round, oval or slightly irregular nucleus with one or two

10 new born animals were inoculated on 6 successive days each day with 0 5 ml of sarcoma suspension Sixteen adult guinea pigs were injected with 1 ml of sarcoma suspension 1 5

Twelve guinea pigs were inoculated with a suspension of chicken sarcoma elicited by the Rous virus of the *Mill Hill strain* Six of these were given 0 5 ml the other six 3 ml of a sarcoma suspension 1 5 in Hank's solution with antibiotics

Forty-three new-born guinea pigs were inoculated with a presumedly cell free extract of the Rous sarcoma (*Schmidt-Ruppin strain*) The chicken tumour was finely minced with a pair of scissors, then ground with glass powder for 15 minutes and suspended 1 5 in Hank's solution with antibiotics The suspension was clarified in a MSE super multex centrifuge for 15 minutes at 4 000 r p m The supernatant was transferred to an International cold centrifuge and centrifuged at 13 800 r p m (10 000 g) for 30 minutes at —3° C The supernatant was cautiously removed and re centrifuged for the same time and at the same rate The procedure was repeated once more and the final supernatant obtained was used for inoculation Of the 43 guinea pigs, 19 were given a single injection of 0 5 ml cell free extract, 8 a daily injection of 0 5 ml on 6 successive days, and 8 a single injection of 3 ml of extract Eight guinea pigs were inoculated intraperitoneally on 6 successive days each day with 0 5 ml extract

Finally 6 new born guinea pigs were inoculated with 1 ml of chicken sarcoma extract (Schmidt Ruppin strain) that had been passed through a Seitz filter

Tissue Cultures

Parts of the guinea pig tumours were finely cut with scissors and trypsinized in 0 1 per cent Trypure Novo The cells were cultivated in Carrel flasks containing Parker 199 with 2 per cent pooled inactivated calf serum to which was added 100 U of penicillin and 100 U of streptomycin per ml The medium was changed every other day

Fibroblasts from 30 day old guinea pig embryos were cultivated in the same way and inoculated with Rous virus (Schmidt-Ruppin strain and Mill Hill strain)

Occurrence of Haemagglutinins

HA tests were carried out on blood cells from guinea pigs chickens sheep and human beings using supernatant obtained on centrifugation of homogenized guinea pig sarcoma suspended in saline or on supernatant from sarcoma cells cultured *in vitro* No haemagglutinins were demonstrable HI tests on sarcoma material and blood from guinea pigs harbouring tumours were kindly done by Dr *H O Sjögren* Institute for Tumour Biology, Stockholm The titre of haemagglutinin inhibiting antibodies did not indicate the presence of polyomavirus

Serial Transplantation of the Guinea Pig Sarcoma

Soft vital parts of the guinea pig tumour were minced with a pair of scissors and suspended 1 5 in Hank s solution with antibiotics The coarser particles were allowed to settle One millilitre of the turbid supernatant was injected intramuscularly into the right thigh of guinea pigs less than 2 weeks old

Histological Technique

Guinea pigs with tumours were sacrificed at various periods after inoculation Pieces of the tumours the inguinal retroperitoneal and mediastinal lymph nodes as well as parts of the liver lungs and kidneys were fixed in Susa embedded sectioned and stained routinely with haematoxylin and eosin sometimes with van Gieson s Masson s and Laidlaw's stain for reticulin

RESULTS

1 *Injection of Material from Rous Sarcoma (Schmidt-Ruppin Strain)*

Tumours developed in 18 of the 23 new-born guinea pigs which had received a single injection of the Rous sarcoma suspension (*Schmidt-*

distinct nucleoli and sparse chromatin; the cells were round or of slightly irregular outline and the cytoplasm was most often relatively rich, pale or slightly eosinophilic These two types of cells were often intermingled, but in the harder, fibrous tumours the former dominated, while the softer tumours were built up of free, rounded cells In the latter case the tumour often had the character of an anaplastic round cell sarcoma (Fig 6) More or less numerous connective tissue fibrils, readily recognized on silver impregnation were seen between the cells Sometimes multinuclear giant cells were present, which gave the tumours a very polymorphous appearance (Fig 7) Some tumours showed elongated band-like cells, whose cytoplasm was longitudinally striated (Fig 8) The picture resembled that of a rhabdomyosarcoma, but no cross striation could be demonstrated

The lymph nodes in the neighbourhood of the tumours were sometimes totally or partly invaded by tumour cells No metastases were seen in the retroperitoneal or mediastinal lymph nodes No cystic transformation of the lymph nodes was observed The metastases in the lungs (Fig 9–10 and 10) had the character of a spindle-cell sarcoma and were built up of elongated cells with oval nuclei In many metastases the cells surrounded the bronchi like a cuff, but sharply demarcated collections of sarcoma cells were also seen in the lung parenchyma Central areas of necrosis and inflammatory infiltrates were seen in the larger tumour nodules in the lungs No secondary growths could be detected in the liver, spleen or kidneys

All attempts to induce sarcomas in new born guinea pigs with Seitz filtrates of extracts of the chicken sarcoma (Schmidt-Ruppin strain) failed

2 *Serial Transfer of the Guinea Pig Sarcoma*

Six 1 2 week old guinea pigs were inoculated intramuscularly with 0 5 1 ml of a 1-0 week old guinea pig tumour suspended 1 5 in Hank's solution with antibiotics Within 9 days 4 of the injected animals developed a 2 × 2 × 2 cm tumour at the site of infection These tumours rapidly increased in size The tumours were soft and their cut surface was greyish-white and moist No metastases were seen Pieces of one of the tumour were finely minced and suspended 1 5 in Hank's solution One millilitre was injected intramuscularly into the right hind leg of 5 guinea pigs weighing about 250 grams Takes were obtained in

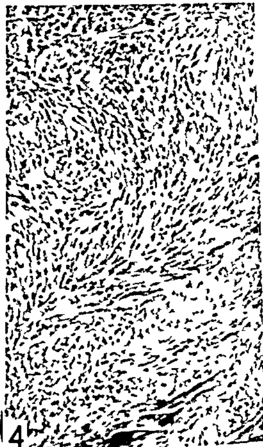
Fig 1 Ulcerated tumour in the hind leg 6 weeks aft [illegible]

Fig 2 [illegible]

Fig 3 [illegible]

Fig 4 [illegible]









Figs 1 4



Figs 9–10

Fig 9 Metastases in the lungs of a guinea pig inoculated intramuscularly in the right hind leg with material from Rous chicken sarcoma × 15

Fig 10 The metastases in the lungs are built up of closely packed spindle cells Htx eosin × 130

3 of them, in which the tumours attained the size of 3 × 3 × 2 cm within 10 days (Fig 11) Material from one of the tumours was transferred to 4 guinea pigs weighing 300–400 grammes Takes were obtained in 2 animals, but the growth of the tumour was slower than before On further passage to 4 new guinea pigs only temporary growth was obtained and the tumours regressed after a week

During the first passages all guinea pigs inoculated with the tumour died The liver often showed more or less extensive necroses and one of the animals had focal necroses in the spleen Treatment of the animals with chloromycetin did not ward off the fatal issue

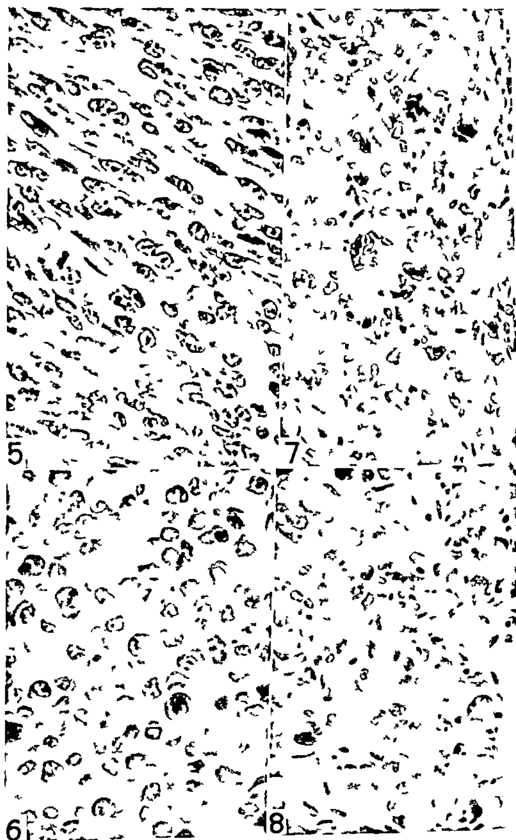
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Fig 5 Two types of cells can often be distinguished in the guinea pig sarcoma one with a narrow nucleus fairly rich in chromatin another with a round or oval nucleus with sparse chromatin and one or two distinct nucleoli Htx eosin × 480

Fig 6 Guinea pig sarcoma with the appearance of a round cells sarcoma Htx eosin × 480

Fig 7 Guinea pig sarcoma of pleomorphic character Htx eosin × 350

Fig 8 Guinea pig sarcoma with numerous elongated cells whose cytoplasm was longitudinally striated *Htx -eosin × 400*



Figs 5–8



Figs 9 10

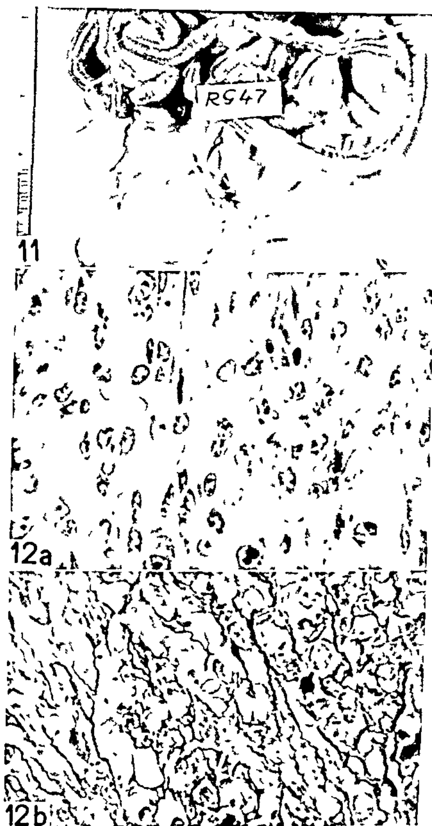
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Figs 11–12

Histologically the tumour in the first guinea pig was an anaplastic medullary sarcoma built up of slightly irregular cells with oval or round nuclei and a sparse cytoplasm (Fig 12) On silver staining a network of argentophilic fibrils was seen between the cells Numerous mitoses were observed The structure was maintained during the successive passages apart from an admixture of leukocytes in some of the tumours The number of mitoses in the material used for the last passage was still rather high

Several attempts to transplant guinea pig tumours of spindle cell sarcoma type have so far failed

All attempts to transfer the guinea pig sarcoma to guinea pigs by means of supernatant fluid from centrifuged guinea pig tumour homogenates suspended in Hank s solution have proved unsuccessful

3 *Back Transfer of the Guinea Pig Sarcoma to the Chickens*

In a series of guinea pigs 0 5 ml chicken sarcoma suspension was injected into the right thigh One week later the injected thigh muscles of one of the animals were excised and saline extract from the excised muscles was injected into the breast muscles of 4 chickens All of them developed typical Rous sarcoma at the site of injection and metastases in the lungs liver and heart Another guinea pig was killed one month after the inoculation at which time it had a 2 5 × 2 5 × 2 cm tumour in the thigh 0 5 ml of a finely minced suspension of the tumour was injected bilaterally into the breast muscles of 3 chickens One of these developed a sarcoma of Rous type on one side of pectus carinatum No tumour appeared on the other side or in any of the other inoculated chickens All attempts to transfer the tumour to chickens with the use of material from guinea pig tumours older than one month calculated from the date of inoculation of the animal with chicken tumour material have failed Nor have repeated attempts to transfer the passage guinea pig tumour to chickens met with any success

4 *Injection of Material from Rous Sarcoma (Strain Mill Hill)*

The Rous sarcoma of Mill Hill strain was passed at 2 week intervals through a series of 3 week old chickens A saline suspension of the tumour prepared in the same way as the material from Rous sarcoma of Schmidt Ruppin strain was given to 12 newborn guinea pigs (150 160 grammes) No tumour appeared in any of the inoculated animals They were observed for 4 months

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Fig 11 Second passage of a transplanted guinea pig sarcoma

Fig 12 The transplanted guinea pig sarcoma had a rather anaplastic character Sparse argentophilic fibrils were seen between the sarcoma cells A Htx eosin B Laidlaw s stain × 480

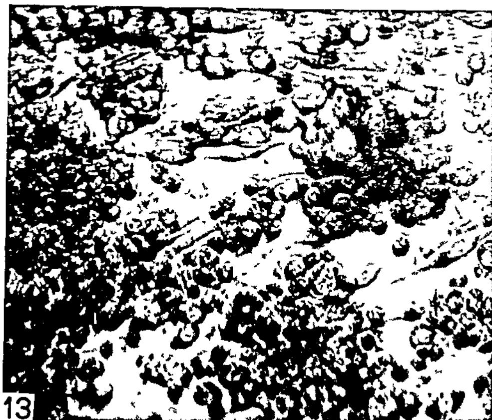


Fig 13

Multilayered foci of rounded cells with granulated cytoplasm appearing in cultures of embryonal guinea pig fibroblasts inoculated with Rous chicken sarcoma virus (*Schmidt Ruppin strain*)

5 *Tissue Culture Experiments*

Cells isolated from the guinea pig tumours and dispersed in trypsin grew during the first week as a monolayer Then multilayered foci of rounded cells with granulated cytoplasm appeared (Fig 13) Similar plaques appeared after 9 days in cultures of embryonal guinea pig fibro blasts inoculated with Rous virus (Schmith-Ruppin strain)

No plaques appeared in cultures of embryonal guinea pig fibroblasts inoculated with the Mill Hill strain of Rous virus

DISCUSSION

Various investigators have tried to transplant Rous chicken sarcoma to the anterior chamber of the eye (14) or to the brain (1, 9) of guinea pigs Only temporary growth was obtained No growth was seen in our experiments in which cellular suspension of Rous sarcoma of Mill Hill strain was transferred to new born guinea pigs

In contradistinction hereto tumours developed in about 75 per cent of the guinea pigs inoculated with material from Rous sarcoma of Schmidt-Ruppin strain For several reasons we think that these tu-

mours are not the result of proliferation of the implanted cells but induced *de novo* by the effect of some agent in the tumour material. Heterotransplantation of cancer cells is difficult unless the innate resistance of the recipients is modified by treatment with x-rays, cortisone or agents having similar effects. No such treatment was given to our animals. Tumours were obtained not only after transfer of cellular suspension of the chicken tumour, but also after injection of a presumably cell-free extract of the tumour. Preliminary chromosome analysis of the guinea pig sarcoma cells by ***Professor Albert Levan*** showed chromosomes of guina pig type. No cells with chromosomes of bird-type were observed. The oncogenic effect on rats, mice, hamsters and rabbits of Rous sarcoma virus (Schmidt-Ruppin strain), has been demonstrated in previous investigations (2–7, 11). In rats no serological evidence of any antigen common to the rat sarcoma and the chicken sarcoma could be found (5, 10). The chromosomes of the sarcomas in rats and mice had the character of rat and mouse chromosomes, respectively (5, 11, 12). It was concluded that the tumour in rats, mice and hamsters were built up of the hosts' own cells and that the tumours had been induced by a virus. There is no reason to assume that the oncogenic mechanism in the guinea pigs should differ from that in the other mammals.

The nature of [illegible]
a variant (mutan [illegible]
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from Rous sarcoma of Mill Hill strain and of Schmidt-Ruppin strain,

[illegible] presence of polyoma virus have been found in the Rous tumour carrying mammals or in chickens inoculated with the Rous virus. The latency, i.e. the time between the inoculation of the virus and the first palpable tumour, is much shorter in Rous virus infected guinea pigs than after infection with polyoma virus. Inoculation with polyoma virus will induce tumours also in the liver, adrenals, kidneys and spleen, i.e. in organs never found to harbour neoplasms in our guinea pigs. Apparently unlike polyoma virus induced neoplasms Rous sarcomas in guinea pigs sometimes regress spontaneously. The structural resemblance between the two types of virus tumours in guinea pigs might be due to the connective tissue reacting in the same way to different oncogenous viruses.

The guinea pig tumours and the tumours obtained in the other mammals inoculated with Rous virus (Schmidt-Ruppin strain) are similar

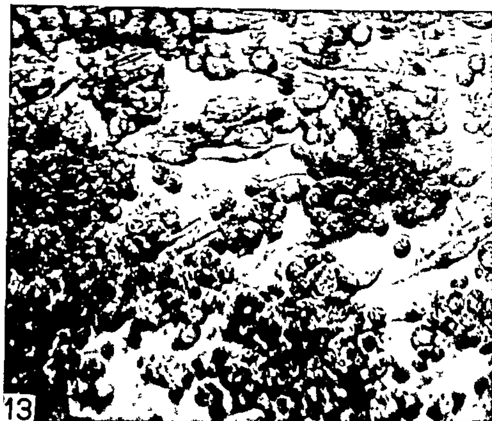


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The nature of the causal agent is still unknown but it is most likely a variant (mutant) of the classical Rous sarcoma virus. Neutralization tests carried out with anti serum from rabbits immunized by material from Rous sarcoma of Mill Hill strain and of Schmidt-Ruppin strain, respectively, indicate that the virus strains are closely related but not identical (2). However, the possibility of contamination of the chicken sarcoma with some other virus such as SE polyoma virus must also be considered, especially as the spectrum of susceptibility in mammals seems to be the same for both viruses. In guinea pigs the polyomavirus induces, at the site of inoculation, undifferentiated sarcomas, composed mainly of spindle shaped cells (8). However, no HA or HI antibodies indicating the presence of polyoma virus have been found in the Rous tumour carrying mammals or in chickens inoculated with the Rous virus. The latency, i.e. the time between the inoculation of the virus and the first palpable tumour, is much shorter in Rous virus infected guinea pigs than after infection with polyoma virus. Inoculation with polyoma virus will induce tumours also in the liver, adrenals, kidneys and spleen, i.e. in organs never found to harbour neoplasms in our guinea pigs. Apparently unlike polyoma virus induced neoplasms Rous sarcomas in guinea pigs sometimes regress spontaneously. The structural resemblance between the two types of virus tumours in guinea pigs might be due to the connective tissue reacting in the same way to different oncogenous viruses.

The guinea pig tumours and the tumours obtained in the other mammals inoculated with Rous virus (Schmidt Ruppin strain) are similar

in many respects They all appear at the site of injection and after a relatively short period of latency Histologically they often have the character of spindle cell sarcoma Two types of cells, one spindle shaped the other rounded or irregular "macrophage"-like has been distinguished in rat, mice, hamster and guinea pig tumours This is noteworthy because two types of cells principally similar to those in the mammalian tumours can also be distinguished in Rous sarcoma in chickens

On the other hand, in guinea pigs the reaction to the Rous virus was not identical with that in rats, mice and hamsters Common findings in rats were cysts, usually situated in the groin, axillae and neck and most often filled with haemorrhagic fluid (2, 3, 5, 15–18) Similar lymphogenic cysts have been seen in rabbits (19, 20) and occasionally in hamsters (personal observation) No cysts were seen in the guinea pigs The sarcomas often metastasized in the rats, mice and hamsters, but rarely in the guinea pigs In the former animals the tumours always grew progressively, in guinea pigs they sometimes regressed In the rabbits the tumour always disappeared within some weeks It thus appears that the reaction to the Rous virus is fundamentally the same in the different mammals but differs in some respects from species to species

The sarcomas could be readily carried in series through rats, mice, and hamsters respectively (1–6, 15 18) The tumour could also sometimes be successfully transplanted in series in guinea pigs, but not through more than three passages It is possible that the guinea pig tumour has less growth energy than the tumours in the other mammals The rats, mice, and hamsters all belonged to a closed colony kept at the institute for a long time, whereas the guinea pigs were purchased from various dealers They therefore possibly had a more heterogenous character than the other animals and this might also influence the growth of the transplanted tumour

On back transfer of the rat, mice, and hamster sarcoma to chicken a tumour developed at the site of injection, which had the usual appearance of a Rous sarcoma (1 6, 15–16) The same result was obtained after injection of material from Rous tumours passed in series in the above-mentioned mammals Thus, in our laboratory the Rous virus has been recovered from the 72nd passage tumour in rats, the 47th passage tumour in mice and the 7th passage tumour in hamsters *Svoboda* (18) has recovered his Rous virus from rat sarcomas passed through 45 generations But all attempts to recover the virus from Rous guinea *pig tumours* carried in series in guinea pigs failed In guinea pigs injected with Rous virus the virus was found at the site of injection one week after the inoculation Its amount or activity was substantially reduced one month after the inoculation (in a 12 day old guinea pig tumour) In spite of numerous attempts the virus could not be demonstrated in older guinea pig tumours

Not even injection into chickens of material from guinea pig tumours which had been grown in tissue culture for several weeks and in which the increase of the virus had not been hampered by antibodies was followed by a tumour

The failure to recover the virus from the guinea pig tumours raises many questions One might imagine that the virus is present in a latent or inactive form or in too small an amount to elicit tumours in a new animal It is also possible that the virus only initiated the guinea pig tumours and that the further growth of the tumours occurs independently of the virus Answers to these and related questions must abide future research

SUMMARY

New born guinea pigs were inoculated intramuscularly with a suspension of finely minced Rous chicken sarcoma (Schmidt Ruppin strain) or with presumedly cell free supernatant fluid obtained by repeated centrifugation of suspension of homogenized chicken sarcoma In about 75 per cent of the guinea pigs tumours developed at the site of injection within 3 to 4 weeks They usually grew progressively In a few cases secondary growths appeared in the lungs Sometimes the tumours spontaneously regressed Histologically they usually had the character of spindle cell sarcomas sometimes of round cell sarcomas or pleomorphic sarcomas

The guinea pig sarcoma was successfully transplanted to new born guinea pigs and could be passed in series through 3 generations All attempts to transfer the sarcoma from guinea pig to guinea pig by means of cell free material from the guinea pig sarcoma failed Material from 14 day old guinea pig sarcoma (1 month after the inoculation of the virus) injected into chickens elicited a Rous sarcoma at the site of the injection All attempts to recover the virus from older guinea pig tumours failed

Embryonal guinea pig fibroblast inoculated *in vitro* with Rous sarcomavirus (strain Schmidt Ruppin) developed plaques within 9–10 days No such plaques appeared after inoculation with Rous sarcoma of Mill Hill strain

No tumour appeared in guinea pigs inoculated with Rous sarcoma of Mill Hill strain

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DEPARTMENT OF PHYSIOLOGICAL ZOOLOGY UNIVERSITY OF HELSINKI AND STATE SERUM INSTITUTE HELSINKI FINLAND

VIRAL SUSCEPTIBILITY AND EMBRYONIC DIFFERENTIATION

I The Histopathology of Mouse Kidney Rudiments Infected with Polyoma and Vesicular Stomatitis Viruses in vitro

By

LAURI SAXÉN, TAPANI VAINIO and SULO TOIVONEN

Received 2 1 63

The word "competence has long been used by embryologists to indicate the capacity of cells and tissues to respond to environmental conditions which lead to changes in the expression of their genetic potentialities During normal development, this response may be termed determination under experimental conditions we are as a rule dealing with a change in the prospective determination Because of the lack of specific markers the epigenetic factors studied to date have mainly been factors and conditions which lead to a change in the morphology of the cells and thus belong to the category of "differentiating" factors During foetal life the capacity of the cells to respond to such morphogenetic stimuli undergoes restriction, although in a broader sense most of the cells in an adult organism continue to exhibit some competence The metaplasia of differentiated cells can be taken as one such example, and perhaps the malignant transformation of cells is indicative of a certain competence to respond to environmental factors

As was mentioned above, our present knowledge of the changes in cellular competence is principally based upon stu[illegible]

[illegible] factors accordingly we have initiated a series of experiments with another epigenetic factor, the virus and have analysed the changes in "viral competence" during embryonic differentiation A large amount of clinical data and the results of experiments made by teratologists, have suggested that such changes in viral susceptibility do occur during foetal life (reviewed *Ebert & Wilt*

This investigation was supported by a PHS research grant C-5347 from the *National Cancer Institute Public Health Service* and by a grant from *Sigrid Juselius Foundation*

The authors wish to thank *Mrs Anja Tuomi Miss Ann Kristin Thors* and *Miss Liisa Helleman* for technical assistance

1960, *Tondury*, 1962) These changes have been correlated to certain stages in normal development, but their background and mechanism are unknown Consequently, we have endeavoured to find new examples of such changes, and especially in organs where the development and its normal control mechanisms are well known Earlier reports of the effect of polyoma virus upon certain tissues (*Dawe* 1960 *Dawe et al* 1962) together with the investigations of *Grobstein* (1953 1956 1961) on the development of the kidney rudiment in vitro seemed to provid an opportunity for the initiation of such studies

MATERIAL AND METHODS

Mice Random bred Swiss mice were used Eleven to 16 day embryos were used and the 0 day was evidenced by vaginal plugging

Organ cultures The kidneys of the embryos were removed and placed on a Trowell type metal screen on a Millipore filter (for detailed description see *Saxen et al 1962*) *In some experiments the rudiments were cultivated direct on a collagenized* cover slip (*Vainio* 1959) Eagles basal medium in Earles balanced salt solution was used with 10 per cent calf serum The medium contained 0 0172 M of sodium bicarbonate and the cultures were incubated in 5 per cent CO_2 in air

Viruses The polyoma virus utilized in these studies was prepared in mouse fibroblast cultures The HA titre of the stock virus was 1 1024, when newborn litters of random bred Swiss mice were infected with this virus preparation it caused a tumour frequency of 80 per cent over an observation period of 6 months

Vesicular stomatitis virus (VSV) was obtained through the courtesy of Dr *Karl Cantell* of the State Serum Institute Helsinki It was prepared in a human amnion cell line (U cells) The stock virus had a titre of $10^{8.3}$ pfu/cc when titrated in chick embryo fibroblasts The same stock of VSV stored at —40° was used throughout the experiments

Infection of the cultures The cultures were adsorbed for one hour with either of the viruses 5 cc of viral dilution was used and not more than 20 organ rudiments were placed in this volume The polyoma virus was used in 10 1 dilution from the original stock virus the corresponding dilution of VSV being 10^{3} After adsorption the cultures were *thoroughly washed in phosphate buffered saline to which had* had been added 10 per cent calf serum and thereafter transferred to culture dishes

Microscopy For fixation the rudiments were carefully removed from the filter paper (or the cover slip) and kept for 30 min in neutral 10 per cent formalin The specimens were dehydrated in butanol series embedded in paraffin and serially sectioned Different staining methods were used one of the most suitable seemed to be Mayers haemalum plus periodic acid Schiff stain

Photography In addition to the usual microphotograms the cultures were photographed frequently under living conditions The collagenized cover slips provided excellent optical conditions for this purpose

Experimental series The histopathology of the lesions caused by polyoma infection was followed in 368 kidney rudiments The detailed description of these series has been published earlier (*Saxen et al* 1962)

As regards the series infected with VSV the kidneys mainly used were from 14 day embryos After infection the kidneys were fixed after 18 20 24 32 36 40 and 48 hours three kidneys being investigated on each occasion The series was repeated twice In addition 33 rudiments were used for special stainings after 24 hours cultivation

RESULTS

Normal development The main features of the development of mouse kidney rudiment under our experimental conditions need a *brief de*scription When removed from a 12-day embryos, the metanephro

genic mesenchyme is morphologically undifferentiated, although the typical shape of the metanephros can be seen in the mesenchymal condensation The ureteric bud has already reached the metanephrogenic mesenchyme, and has in most cases formed the first branches Following subsequent cultivation for 24 hours, the ureteric bud has branched twice or three times, and the onset of tubulogenesis is seen as a condensation of the peripheral metanephrogenic mesenchyme (Fig 1) After a total period of cultivation of 48 hours, the first tubuli are seen in gross examination They are located between the peripheral, undifferentiated mesenchyme and the central loose mesenchymal tissue, and are visible as small applelike formations (Fig 2) During subsequent cultivation, new tubuli are continuously being formed between these two areas, and simultaneously a more delayed tubulogenesis is seen in the peripheral parts (Figs 3 and 4) Thus, between 2 and 12 14 days a series of tubules in different developmental stages is seen in all the cultures the youngest condensations are the most peripheral, and the oldest tubuli with definite cytodifferentiation are located in the central portions (Figs 1 to 4) After two weeks of cultivation, the formation of new tubules seems to cease, and the tubuli attain their typical S shape and become connected with the collecting system

Without going into details of the histogenesis, it is important to describe the different tissue components seen in all of these rudiments This is illustrated in Fig 5 The distinction between these tissue componenents must be effected before a study is made of the more or less specific distribution of viral lesion in the kidney rudiments

The histopathology of the polyoma lesions No more than a summarized description of the effect of polyoma virus on the kidney rudiment is presented below the observations have been presented in detail in a previous paper (*Saxen et al* 1962)

The various tissue components mentioned above show definite differences in their viral susceptibility The loose central mesenchyme is affected first, resulting in the appearance of degenerating cells and cell debris between the branches of the uteric bud and the developing secretory tubules (Figs 6 and 7) The condensed areas, along with the kidney tubules at different stages of development, seem to resist the pathogenic effect of the virus for a rather long period When the first degenerative changes in the undifferentiated mesenchyme become discernible two days after infection the epithelial cells are still morphologically normal after a subsequent cultivation of 10–12 [illegible]

[illegible] to judge whether we are dealing with a direct effect of the virus, or whether the changes can be attributed to the unsuitable environmental conditions provided by the lytic tissue

As concerns the cytology of the viral lesions caused by polyoma virus, not much can be added to our very scanty information on viral cyto-


1
2


3


4

Figs 1–4

Four developmental stages of mouse kidney rudiment cultivated *in vitro*, and photographed in living conditions on a collagenized cover slip Rudiments were removed from 12 day embryos and cultivated for one (Fig 1), three (Fig 2), seven (Fig 3), and 10 days (Fig 4) respectively The onset of tubulogenesis is seen as condensation f peripheral metanephrogenic mesenchyme and the secretory tubuli appear as sharply demarcated balls in the more central parts of the rudiments in Figs 2 4

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pathology In addition to nuclear pycnosis leading to complete disintegration of the nuclei and to the appearance of Feulgen-positive debris, nuclear "ballooning" and inclusion bodies are seen in the mesenchymal cells Furthermore, paranuclear vacuolization is noted in some cells, especially in the epithelial cells of the old, disintegrating cultures

The histopathology of the VSV lesions The present series of microphotographs gives us an idea of the main features of the lesions caused by VSV under our experimental conditions Against what was said above relating to the CPE of the polyoma virus, the higher pathogenicity of the VSV is obvious first lesions were obtained after 18–20 hours, and in all the series the kidneys were completely *disintegrated* after 48 hours of cultivation These observations were made in 14-day

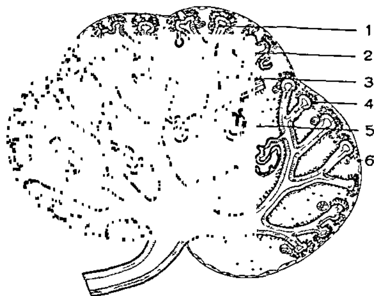


Fig 5

mesenchyme ... capsular

16

7

kidneys, when younger rudiments from 12 day embryos were used, the destruction was even more rapid

As was the case in polyoma-infected kidneys, definite differences in the susceptibility of different tissue components became evident—although in a somewhat different way The first lesions were now observed in the *condensed mesenchyme* around the ends of the collecting tubes (Figs 9, 10, 14, 15) This was observable in both the peripheral condensations and the older, central pre-tubular condensations which did not show epithelial differentiation In contrast, the tubular structures and the branches of the collecting system seemed to resist the viral effect at this stage of infection (18 to 30 hours subsequent to infection) At this time the loose mesenchyme between the condensed areas gradually became affected, whereas the mesodermal sheath around the collecting tubes was morphologically intact Subsequently, the secretory tubules also showed the first degenerative changes, but here again, not before they had been surrounded by dead or dying mesenchymal cells At the final stage, even the well-differentiated epithelium of the collecting system exhibited degeneration

The cytological changes consist in the main of nuclear pycnosis and disintegration However, when a comparison is made with our observations on the effects of polyoma virus, certain differences are noticeable Nuclear ballooning, described by us, and many investigators before us (*Stewart* 1960 *Dawe & Law* 1959, *Stanton et al* 1959), in polyoma-infected cultures is lacking or at least very rare in cultures infected with VSV In contrast, the "paranuclear vacuolation" described by *Weis & Kirsten* (1962) in polyoma infection was quite exceptional in our polyoma cultures, but common in the kidney cells infected with VSV In the secretory tubules surrounded by cell debris in particular, this type of lesion appeared to be the most common (Fig 16)

By way of conclusion, it may be added that similar or other degenerative changes were rare in the control series cultivated under identical conditions without infection On occasion, some dying mesenchymal cells could be seen in the serial sections of the younger cultures In cultures kept *in vitro* for more than 12 days, the central parts of rudiments showed more advanced lesions, obviously due to unfavourable nutritional conditions However, these lesions were clearly distinguishable from the viral cytopathic effects

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Fig 6 and 7

Two kidney rud— — lesions consist

The un ecre cul rudiment cultivated and periodic acid—



Figs 8–13

A series of microphotographs showing the viral lesions in a 14 day mouse kidney rudiment infected with vesicular stomatitis virus (VSV) and subsequently cultivated for 20 24 28 32 36 and 40 hours The first lesions are seen in the peripheral condensations and subsequently in the stromal mesenchyme Not until 10 hours later are the secretory tubuli affected and even after 36 to 40 hours cultivation subsequent to the infection the collecting tubes and their mesenchymal sheath seem morphologically normal *Haematoxylin and periodic acid—Schiff* × 200

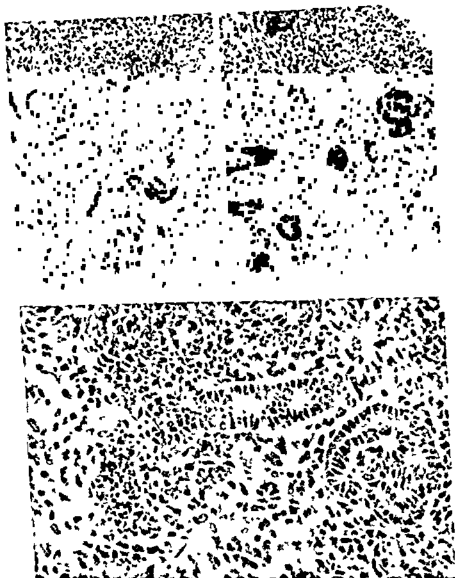
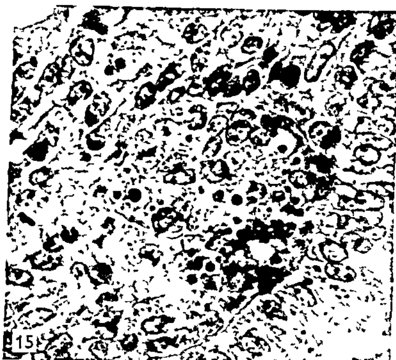
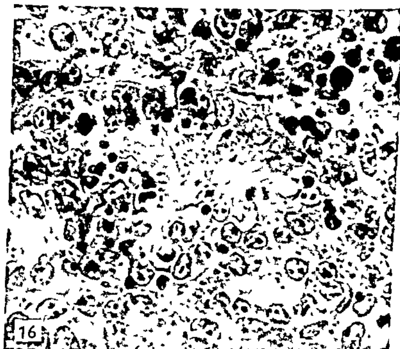


Fig 14

A detail of a 14 days kidney rudiment infected with VSV and subsequently cultivated for 22 hours From right to left the figure shows the main components in the nephrogenic tissue a secretory tubulus a collecting tube with its two branches metanephrogenic condensation around the tips of these branches and the loose stromal mesenchyme in between these components The viral lesions are seen in the mesenchymal condensations Haematoxylin and eosin × 350





Figs 15 and 16

Fig 15 A typical early lesion by VSV in a mesenchymal condensation *close to the* collecting tube Pycnotic nuclei and nuclear debris (pseudoinclusions) are to be noted Haematoxylin and periodic acid—Schiff × 700

Fig 16 Late viral lesions in a kidney rudiment infected with VSV The secretory tubulus is surrounded by cellular debris and the epithelial cells of the tubulus show degenerating nuclei and paranuclear vesiculation Haematoxylin and periodic acid Schiff × 700

DISCUSSION

The results presented above show that there are definite differences in the viral susceptibility, the "viral competence" of the embryonic cells in one and the same organ rudiment. Furthermore, they show that changes in this susceptibility do occur during development. Metanephrogenic cells are highly susceptible before the onset of cytodifferentiation, but resist the viral CPL after getting involved in the tubulogenesis. In addition, the results indicate that there are already agent-specific differences present in the viral susceptibility at an early stage of development: the condensed, pretubular mesenchyme seems to resist the CPL of polyoma virus, but is highly susceptible to the pathogenic effect of vesicular stomatitis virus. It is unnecessary to stress that this study constitutes but a very limited example of viral effects on embryonic tissues, and represents no more than the start of a more extensive series of investigations along these lines. Moreover, this part remains purely descriptive in nature, and follows the classical lines of histopathology. Nevertheless some questions can be brought up in this connection and we should like especially to compare our results with the present knowledge of experimental teratology.

In recent years, experimental teratology has yielded abundant evidence for the classical concept of *Stockard* (1921) which states that foetal lesions due to environmental factors are largely dependent upon the developmental stage of the pathogenic treatment: different teratogenic agents result in similar anomalies when applied at the same stage of development, whereas the same factor leads to different types of lesions at different stages of foetal development.

The importance of the stage of development of teratogenic treatment has been demonstrated in many investigations concerning foetal lesions brought about by viral infections. The voluminous literature on the effect of maternal rubella on the human foetus indicates that the child can be damaged only when the disease is contracted during the classical "sensitive period", the first trimester of pregnancy. Furthermore, the localization of the viral lesion (lens primordium, ear vesicle, heart, etc.) seems to depend on the time of the rubella infection (reviewed by *Swan* 1946, *Mayes* 1957, *Siegel & Greenberg* 1960, *Tondury* 1962). Experimental results with different viruses have confirmed these ideas.

In a number of experiments with influenza A virus, stress has been laid on the importance of the time of infection (*Hamburger & Habel* 1947, *Williamson et al.* 1953). The observations of *Kuna* (1918 ... *Burnet* (1940 ...
former ...
ceptible ... virus, whereas the latter demonstrated that some 10 days later this susceptibility was restricted to the lung primordium. Corresponding observations have been reported from experiments with Newcastle disease virus (*Blattner & Williamson* 1951, *Williamson et al.* 1953) and with vaccinia virus (*Thalhammer* 1957).

When the different factors responsible for such "sensitive periods" for viral damage are under discussion, a great number of conditions have to be considered. The penetration of the virus into the foetal tissues is naturally of great importance, and it has been suggested that tissues are affected when they are exposed to the exterior at the time of inoculation (*Saxén* 1958, *Ebert* 1961, *Hamburger* 1961). The state of the target tissue has also been mentioned as an important factor. As in discussion of the sensitive periods of other teratogenic factors, the metabolic state and the proliferation rate of the infected tissue have been put forward as having some bearing on the viral susceptibility (*Williamson et al.* 1953, *Robertson et al.* 1955). However, as *Ebert* & *Wilt* (1960) have pointed out, all direct evidence in favour of this "general feeling" is lacking as far as viruses are concerned.

In referring to the complicated problem of viral embryopathies, *Ebert* (1961) recently stated: "There is a compelling need for systematic studies and comparisons of the effects of inoculations of specific viruses into the embryo of successive intervals during ontogeny, under uniform conditions of dosage and other controllable conditions". We do not think that our experimental conditions can meet all the prerequisites he states as necessary, but nevertheless it seems that such *in vitro* studies on organ rudiments can give us a valuable tool for certain comparative investigations under controlled conditions. Of course, this does not imply that results obtained *in vitro* are applicable as such to *in vivo* conditions, as was pointed out in our earlier report (*Vainio et al.* 1963).

In pursuance of the old statement by *Stockard* (1921), it can be concluded that the idea of certain sensitive periods is valid even at a cellular level, as is evidenced by the changes in the susceptibility of the mesenchymal cells in the metanephrogenic anlage. However, our results indicate that the quality of the teratogenic agent cannot be neglected: there were marked definite differences in the cellular specificity of the effect of the two viruses so far studied. The causes of these differences are hardly attributable to the experimental conditions, which were identical in the different series, and thus explanation of the time-dependent and agent-specific susceptibility must be sought in the target tissue itself. Such factors as the presence or the absence of virus receptors, the capacity to support viral replication, the rôle of the metabolic activity and the proliferation rate, etc., need investigation. Our experimental conditions seem to afford an opportunity to do so, and some results of investigations along these lines will be presented in the next papers of this series.

SUMMARY

The paper deals with the histological changes noted in mouse kidney rudiment *in vitro* after infection with SE polyoma virus and vesicular stomatitis virus (VSV). The microscopical lesions are described at the different intervals of the *in vitro* cultivation of infected rudiments

Both stage-dependent and agent-specific features were noted in the viral susceptibility of the different tissue components The rather slow polyoma infection started from the undifferentiated stromal mesenchyme, and both the condensed, pretubular areas and the differentiated secretory tubules seemed to resist the infection VSV first affected the condensed areas of metanephrogenic mesenchyme, and subsequently spread throughout the kidney rudiment

The observations are discussed in the light of viral teratology and earlier observations in vivo The present material and the controlled experimental conditions seem to offer a suitable tool for a better understanding of the basic mechanism of viral infections in embryonic tissues However, no correlation of the present observations to the events of normal morphogenesis can be effected until the normogenesis is more thoroughly analysed

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VIRAL SUSCEPTIBILITY AND EMBRYONIC DIFFERENTIATION

2. *Immunofluorescence Studies of Viral Infection in the Developing Mouse Kidney in vitro*

TAPANI VAINIO, LAURI SAXÉN and SULO TOIVONEN

Received 2.1.63

It was inferred from the experiments presented in our previous paper that vesicular stomatitis virus (VSV) attacks the developing mouse kidney *in vitro* and damages the mesenchymal condensation areas in particular (*Saxén et al.* 1963). This is sharply opposed to the CPE by polyoma virus in the same organ *in vitro* (*Saxén et al.* 1962). The condensation areas seem to be resistant to the CPE of polyoma virus. However, it must be emphasized that VSV is a toxic virus, and may destroy the tissues in which it does not replicate (*Cantell et al.* 1962). Thus the CPE found in the mesenchymal condensations in VSV infection does not necessarily indicate that the virus replicates there, which does in fact determine the susceptibility to a given virus.

To gain some knowledge of which tissue components in the developing mouse kidney support the replication of VSV, some investigations were made with the aid of fluorescent antibodies which had been successfully used in studies with polyoma virus (*Vainio et al.* 1963). In conjunction with this, some comparisons were made between the foetal kidneys infected with VSV or polyoma virus *in vitro*, with the distribution of the viral antigens being taken into consideration.

METHODS

Organ cultures were prepared in the way previously described (*Saxén et al.* 1962) with the culture medium given in the previous paper.

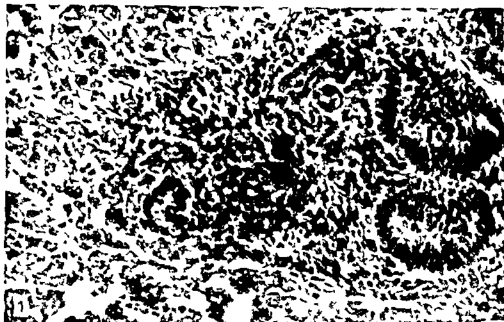
Viruses. The VSV and polyoma virus utilized in these studies were from the same stock as those described in the previous paper.

Infection of cultures. Organ rudiments were adsorbed with VSV and polyoma virus using the dilutions and procedures given earlier.

Immunofluorescence. Antisera against polyoma virus were prepared in rabbits

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The authors wish to thank Mrs. Anja Tuomi, Miss Ann Kristin Thors and Miss Liisa Helleman for technical assistance.





Figs 1 and 2

The section and the corresponding fluorogram of a foetal mouse kidney infected with polyoma virus and cultivated for 6 days *in vitro*. Note the stained nuclei in the cells between the tubuli and the absence of fluorescent material from the tubuli cells themselves

by weekly injections of the stock virus (*Vainio et al* 1963). The intraperitoneal route month of immunization the virus was given ant. The conjugation to fluorescein isothio *arshall et al* (1958). Rabbit acetone powder

was acquired through the courtesy of *Dr Kurt Paucker* the Children's Hospital of Philadelphia USA. It had been prepared



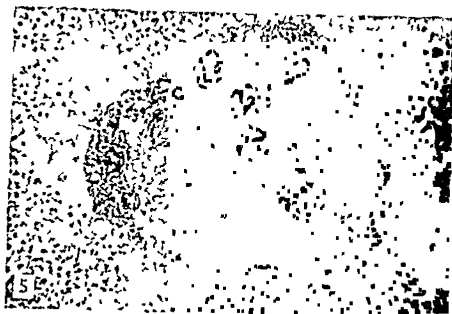


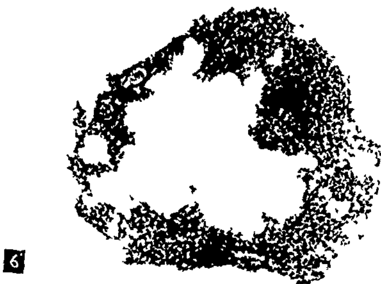
Figs 3 and 4

The section and its fluorogram of a 14 day old foetal mouse kidney infected with VSV and subsequently cultivated for 22 hours The periphery of the organ shows an abundance of the antigen some tubuli remain unstained as do the mesenchymal cells in the middle of the organ

n rabbits and conjugated to fluorescein isothiocyanate (*Paucker et al* 1962) The absorption with rabbit acetone powder was done shortly before the conjugate was to be used

[illegible] hour after which





Figs 5 and 6

The sectic n and the fluorogram of a 14 day old f etal mouse kidney infected with VSV and cultivated f r 26 hours The viral antigen (ccupies the mesenchymal con densation area but the tubuli and the mesenchyme in the mi ldle remain unstained

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Figs 7 and 8

A more detailec , i ture of a section of foetal kidney infected with VSV and culti vated *for 10 h urs al ng with the corresp onding flu rogram* Note that the viral antigen appear n the cells thr ughout the organ l ut yet the collecting system an l its neighbour ng mesenchyme as well as the c nvolute l tul uli are free fr m antigen



the sections were stained with fluorescent conjugate or stored at —40° The mounting medium was either buffered glycerol or Elvanol (Dupont)

After fluorescent microscopy, the coverslips were removed in glycerol and the sections stained with haematoxylin eosin or haematoxylin p.a.S to reveal the structure containing the fluorescent material

Experimental series 13- and 14-day-old foetal mouse kidneys were infected with polyoma virus, sectioned after 2 4, 6 and 8 days and stained with fluorescent antibodies Accordingly, fourteen day-old foetal mouse kidneys were infected with VSV and when they had been cultivated for 16 hours a couple of organs were removed every fourth hour and sectioned and stained with the appropriate fluorescent antiserum The VSV studies were repeated twice, and 55 foetal kidneys were included in the experiment

RESULTS

Polyoma infection A characteristic of the infection caused by polyoma virus was that the viral antigen appeared in the loose mesenchymal tissue in the central area of an infected kidney (*Vainio et al* 1963) While the infection was advancing, the antigen appeared in the mesenchymal cells, in between the tubuli (Figs 1 and 2) However, no virus antigen was detected either in the collecting system or in the renal tubuli Furthermore, the mesenchymal, pretubular condensations did not seem to produce viral antigen upon infection, and thus the cells forming these condensations, behaved differently from the other mesenchymal cells in the cultures

VSV infection In VSV infected organ cultures, the viral antigen appeared first in the periphery It soon occupied a rather deep tissue layer spreading inwards between the renal tubuli, which remained free of antigen (Figs 3 and 4) Within a few hours, the infected area in the periphery of cultured organ had deepened, although tubuli remained free from viral antigen (Figs 5 and 6) The area now occupied by viral antigen was rich in mesenchymal condensations, indicating their susceptibility to antigen production When the synthesis of viral antigen could be observed in the central area of the culture, the renal tubuli appeared relatively free from antigen, as did the whole collecting system (Figs 7 and 8) It was also repeatedly noted that the mesenchyme adjacent to the collecting tubes did not seem to produce viral antigen when the infection had spread through the culture

Polyoma versus VSV infection When infections caused by polyoma virus and VSV are compared, there are some obvious differences in the distribution of the viral antigen The mesenchymal condensations (see Fig 5 in the preceding paper) support the synthesis of VSV antigen, whereas they appear incapable of manufacturing polyoma antigen during infection On the other hand, both collecting and convoluted tubuli appeared to be poor soil for the synthesis of these two viruses, or at least of their antigens The undetermined mesenchyme (Fig 5 in the previous paper) was susceptible to the synthesis of polyoma virus as well as VS virus antigens, whereas the mesenchyme next to the collecting tubes supported the synthesis of polyoma virus antigen but appeared to be resistant to the synthesis of VSV antigen Finally, the

spreading of the infection when caused by polyoma virus was found to be different from that caused by VSV The former tended to start in the central portion of the culture and the latter regulary from the periphery

SUMMARY AND CONCLUSIONS

The results presented above show that the distribution of VSV antigen in foetal mouse kidney *in vitro* closely corresponds that of the histologically observed viral lesions Furthermore this study supports our earlier suggestion of certain differences in the tissue competence (susceptibility) towards the two viruses (VSV and polyoma virus) employed in our studies so far Attention was focused on changes during tubulogenesis where three morphologically distinct stages can be seen *Undifferentiated metanephrogenic mesenchyme → mesenchymal condensations → epithelial tubules* The mesenchyme seems to support the replication of both viruses employed whereas renal tubules appear to be resistant to these viruses Mesenchymal condensations which represent the first detectable step in tubulogenesis resist polyoma virus but are highly susceptible to VSV and support its replication

It may be concluded in this connection that the induction process interferes with the susceptibility of a given tissue to a particular virus However the tissue can remain susceptible to some other viruses Processes relevant to differentiation such as metabolic changes in the differentiating cells have a bearing on viral susceptibility Some of these views will be discussed further in our forthcoming paper

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THE CYTOLOGY OF THE ADRENAL CORTEX IN MICE WITH SPONTANEOUS ADRENOCORTICAL LIPID DEPLETION[1]

By

KRISTEN ARNESEN

Received 1 xii 62

A spontaneously occurring lipid depletion of the adrenal cortex in mice of the AKR/O strain has been described by *Arnesen* (1955, 1956) The lipid depletion takes place at the time of puberty and depends upon the presence of the gonads both in males and females Prepubertal gonadectomy prevents the lipid depletion from taking place

In adult males the lipid depletion is almost total in terms of sudanophilia Occasionally a narrow rim of sudanophilic material may be found in the outer, immediately subcapsular part of the cortex In females the depletion is subtotal, and the lipid pattern of the cortex shows considerable variation

Large doses of ACTH administered to adult AKR/O males restore the lipid concentration and distribution in the adrenal cortex to almost normal

The adrenocortical lipid depletion is due to the effect of one recessive gene, and the following designations have been introduced *The adrenocortical lipid pattern gene*, and *the adrenocortical lipid depletion gene*, which latter is the recessive allele, responsible for the strain specific adrenocortical lipid depletion

The AKR/O strain exhibits a high incidence of lymphatic leukaemia In addition to the particular constitution of the adrenal cortex, this strain is also characterized by a thymus hyperplasia which is manifest from the period of infancy In hybridization experiments some sort of an association between the adrenocortical lipid depletion and the thymus hyperplasia was demonstrated, but the character of this association could not be elucidated

Because of the well known effect of adrenocortical hormones upon the thymus, it was tentatively assumed that the thymus hyperplasia might disclose a state of constitutional hypofunction of the lipid-depleted adrenal cortex It was also discussed as a hypothesis that the specific "adrenothymic constitution" might in some way be associated with the

1 The work has been supported by *Grosserer N A Stangs legat til kreftsykdommers bekjempelse*

innate disposition for lymphatic leukaemia in this strain No definite answers have as yet been given to these questions *Metcalf* (1960), who worked with the same strain of mice and confirmed the finding of the adrenocortical lipid depletion as well as the thymus hyperplasia, has also postulated that the adrenal cortex of these mice is hypofunctional

To my knowledge the adrenocortical lipid depletion as a spontaneously occurring phenomenon, has only been described in the AKR strain (AKR O designating the descendent line kept in Oslo)

From the observations referred to, various important problems emanate of which two are in the centre of interest for this author

1) Has the adrenocortical lipid depletion, as well as the associated thymus hyperplasia, anything to do with the inherited disposition to lymphatic leukaemia in the AKR O strain?

2) What is the functional implication—if any—of the adrenocortical lipid depletion?

The first question is beyond the scope of this paper It can only be answered through time consuming hybridization experiments, which are in progress in our laboratory A brief report of some preliminary results will be published in this journal within short time

The second problem is under study from various angles The adrenocortical lipid depletion as such does not permit any definite conclusion as to the functional state of the organ The adrenocortical lipid pattern is a function of

a) The manufacture of lipids—including steroid hormones and their precursors

b) The storage of lipids—including steroid hormones and their precursors

c) The output of lipids—including steroid hormones Under conditions of homeostasis production must equal output, but neither of these is reflected in the amount stored

The morphological picture of permanent adrenocortical lipid depletion may imply

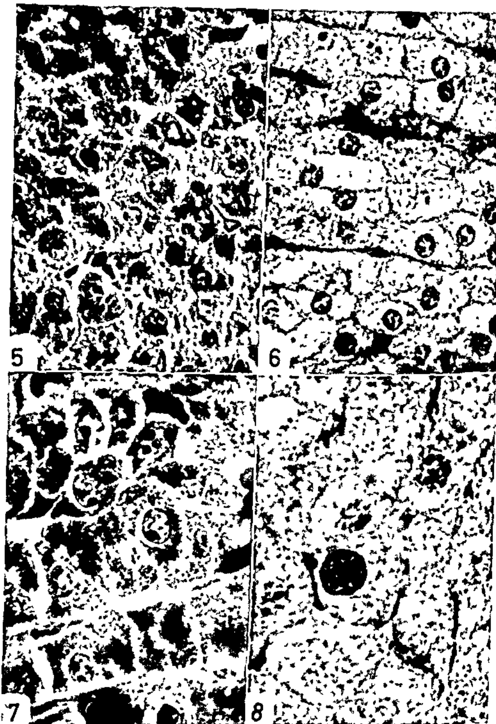
a) Normal output of steroid hormones with normal rate of production but without appreciable storage

b) Increased output of steroids with increased production and no storage

c) Decreased output of steroids with decreased production and no storage

The only thing which can be concluded on the basis of the permanent lipid depletion is that storage is reduced or abolished In this connection it must be pointed out that there is no marked change [illegible]
of the [illegible]

T [illegible]
the [illegible]
fro [illegible]
depletion [illegible] determined adrenocortical lipid



Figs 5–8

Fig 5 CS male 180 days old Cells in zona fasciculata containing numerous mitochondria Iron haematoxylin × 810

Fig 6 WLO male 59 days old Cells in zona fasciculata with vacuolated cytoplasm Iron haematoxylin × 810

Fig 7 Same as Fig 5 × 1320

Fig 8 Same as Fig 6 × 1320

From the Figures 5 and 7 it can be seen that the cortical cells from mice with lipid depletion have a cytoplasm which is tightly packed with small round bodies with a strong affinity to the haematoxylin These usually fill out the whole cell body to the very border of the cell The surface membrane therefore usually appears indistinct In contrast, the cortical cells from the normal mouse (Figs 6 and 8) are faintly stained (light cells, 'helle Zellen), and their cytoplasm seems mostly to consist of closely adjacent vacuoles Mitochondria are very indistinct, and the cell membrane is sharply drawn

As to nuclear structure and size, no clear difference between the two types of adrenal glands have been observed, but exact measurements have not been carried out so far

DISCUSSION

The findings here reported should be interpreted under consideration of the work of *Molbert & Arnesen* (1960), who studied the ultrastructure of the adrenal cortical cells of normal mice, as well as those with spontaneous or arteficially induced lipid depletion In the normal adrenal cortex cells were found with large lipid vacuoles ('Speicherungs locher') between which appeared mitochondria with a rosette like structure In the cortical cells of lipid depleted adrenals the lipid vacuoles were almost completely absent, whereas the mitochondria were abundant, closely packed, showing a characteristic pattern with double contoured *cristae mitochondriales* in a more or less parallel configuration The present study confirms that the cortical cells in spontaneously lipid depleted adrenals are very rich in mitochondria —apparently richer than the corresponding cells of adrenals with a normal cortical lipid pattern These findings are hardly compatible with the concept that the spontaneous lipid depletion reflects a state of permanent hypofunction of the cortex

The term 'function in this connection is equivocal, however One thing is the total or partial function in terms of specific hormone production Another is the basic and unspecific metabolic activity of the cells

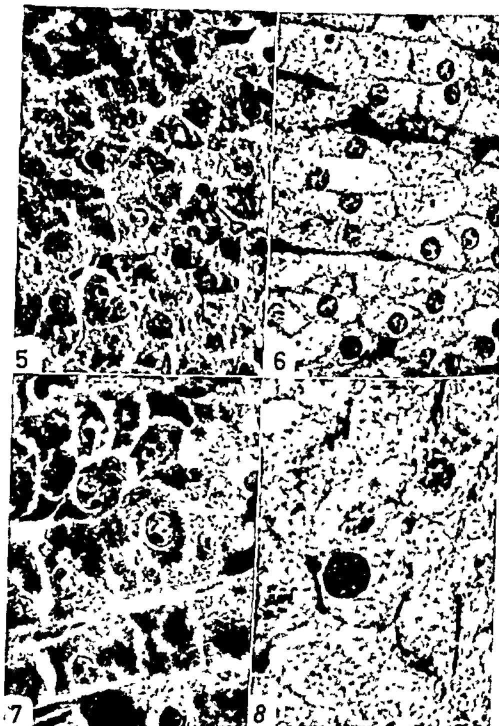
The mitochondria are of paramount importance for the oxydative processes going on in the cells, in so far as they carry the majority of the respiratory enzymes of the cytoplasm Th

and the corresponding variatio

can not yet be interpreted to t

endocrine function of the cortex On the other hand, the observations favour the hypothesis that the basic metabolic (respiratory) activity is high in the lipid depleted adrenal cortical cells —probably higher than in the corresponding lipid laden cells of normal adrenal glan

son (19



Figs 5 8

Fig 5 CS male 180 days old Cells in zona fasciculata containing numerous mito chondria Iron haematoxylin × 810

Fig 6 WIO male 59 days old Cells in zona fasciculata with vacuolated cytoplasm Iron haematoxylin × 810

Fig 7 Same as Fig 5 × 1320

Fig 8 Same as Fig 6 × 1320

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THE EFFECT OF MATERIAL PREPARED FROM OX BLOOD ON COLD STRESS IN MICE

By

J DEDICHEN, P LALAND and S G LALAND

Received 19 xi 62

It has been reported that high molecular material prepared from blood increases resistance *in vivo* to certain bacterial and viral infections (1, 2) These observations indicated that we are dealing with a humoral component of importance to the so called non-specific resistance For this reason it was thought of interest to study the effect of the material under other conditions where resistance could be measured This report describes experiments designed to study the effect of the material in mice exposed to cold stress

MATERIALS AND METHODS

Two materials designated IV and VI prepared from ox blood as described previously were used (1, 2 3)

Swiss albino male mice each weighing 15–18 gr have been used During the experiments the animals were kept in a cold room in two wooden boxes of the same size and each divided into 15 equal square chambers The temperature in the cold room varied from 0 to +2° C Between experiments the empty boxes were always kept in the cold room to secure uniform temperature in all the chambers The mice were without access to food or water during the stress [illegible]

[illegible] the cold stress The [illegible] animals and the controls were distributed in the two boxes at random so that both controls and treated animals were mixed There was one mouse in each chamber and 15 treated and 15 control mice were normally used in each experiment The number of dead animals was recorded every hour for 7 hours

RESULTS

The results obtained using varying amounts of material IV and VI per mouse, are seen in Fig 1–7 The results recorded in each of these comprise a number of experiments

Table 1 gives a survey of the statistical significance of the results recorded in Fig 1 7 It will be seen that [illegible]

adrenal cortex into compact cells under the influence of ACTH or in conditions of prolonged stress, is an expression of increased cellular activity

Studies of cellular metabolism as well as of the production rate and output of hormones are called for in order to reveal the real implication of the spontaneous adrenocortical lipid depletion Investigations along these lines have been taken up in our laboratory

SUMMARY

The spontaneous lipid depletion of the adrenal cortex of mice derived from the AKR/O strain, is determined by the "adrenocortical lipid depletion gene", and is associated with structural changes in the cytoplasm *of the cortical cells, characterized by loss of lipid* vacuoles and the occurrence of great numbers of closely packed mitochondria Cortical cells with a normal lipid pattern are relatively poor in mitochondria, as far as can be judged from the light-microscope pictures The functional implication of the spontaneous lipid depletion is discussed

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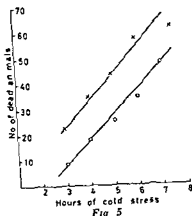


Fig 5

Results of cold stress using 0 084 mg/mouse of material VI 105 mice in each group ——○ treated ×——× controls

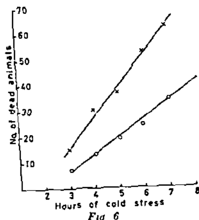


Fig 6

Results of cold stress using 0 168 mg/mouse of material VI 105 mice in each group ○——○ treated ×——× controls.

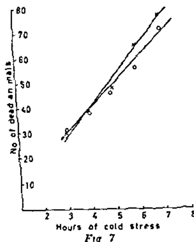


Fig 7

Results of cold stress using 0 672 mg/mouse of material VI 90 mice in each group ○——○ treated ×——× controls

With 96 μg the effect is apparent from 5th hour and the significance increases up to seven hours

Similar results were obtained with material VI which seems less active than material IV since 42 μg produced no protection With 84 μg the protective effect is apparent already after three hours, but disappears after six hours With 168 μg the effect starts after 4 hours and the significance increases up to seven hours were it is very marked At 192 μg of material IV and 672 μg of material VI no effect was apparent

Material IV and VI have been prepared by somewhat different procedures which probably explains the difference in activity

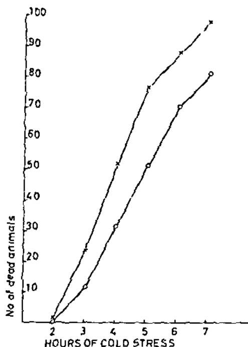


Fig 1

Results of cold stress using 0 018 mg/mouse of material IV
135 mice in each group
O——O treated X ——X controls

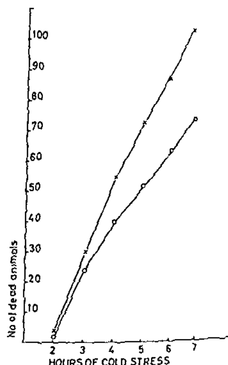


Fig 2

Results of cold stress using 0 096 mg/mouse of material IV
135 mice in each group
O——O treated X——X controls

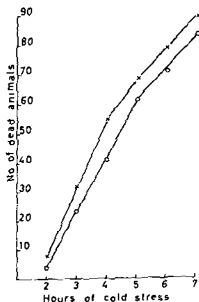


Fig 3

Results of cold stress using 0 192 mg/mouse of material IV.
120 mice in each group
O——O treated X——X controls

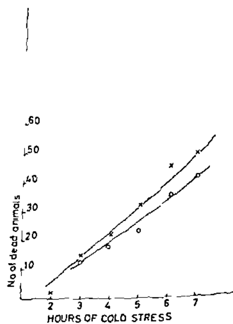


Fig 4

Results of cold stress using 0 042 mg/mouse of material VI
105 mice in each group
O——O treated X——X controls

has been made We have, however, found intestinal haemorrhages to be a common appearance, a phenomenon to which many workers attribute great importance for the development of the irreversible state of shock (13 14) It must be appropriate to characterize the cold stress as a form of shock Our experiments provide no explanation for the effect obtained on the development of experimental shock It seems not unreasonable to identify our material with the hypothetical "resistance factor" which can be released by repeated injections of phenol (15), and probably also by endotoxins when given in appropriate dosage

It is not unlikely that the effect is displayed on a cellular level since it has been found that our materials interfere with the mitotic activity of human cells grown in vitro (3)

For instance the effect may be connected with the intensity of cellular repair

Experiments which intend to study the effect of different quantities of our materials on the mitotic activity in vivo are in progress

SUMMARY

Subcutaneous administration of high molecular material prepared from ox blood 24 hours prior to cold stress in mice significantly reduces the mortality of the animals over a certain dosage range It is suggested that the material is related to a hypothetical resistance factor mentioned in the work of Raskova and Vanecek The effect of the material is probably displayed on a cellular level

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3 *Ofteb* [illegible] The Presence [illegible] the Mitotic [illegible]

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TABLE 1

Statistical Probability for the Significance of the Difference in the Number of Dead Animals in the Treated and Untreated Groups in Experiment 1–8 When P is Greater than 0 05 no Figure Is Recorded

Hours of cold stress	Amount of material IV injected		
	48 µg	96 µg	192 µg
3	0 030	–	–
4	0 006	–	–
5	0 002	0 010	–
6	0 007	0 003	–
7	0 012	< 0 001	–

Hours of cold stress	Amount of material VI injected				
	42 µg	84 µg	168 µg	336 µg*	672 µg
3	–	0 007	–	–	–
4	–	0 006	0 003	–	–
5		0 008	0 004	–	–
6	–	0 001	< 0 001	–	–
7	–	–	< 0 001	–	–

* In this experiment only 60 mice in each group were stressed

DISCUSSION

The interest in the non-specific resistance has lately been stimulated through extensive experimental work on the endotoxins These stimulate the resistance of the organism against infections with bacteria and viruses (4, 5, 6, 7), experimental shock and X-irradiation (8, 9)

The finding that our materials increase the resistance to cold stress over a certain range of dosage whereas higher doses do not produce any effect is interesting and surprising It can hardly be attributed to a toxic effect of the materials which have an LD 50 estimated to 1 9 g/kg mice [1] It is of interest to compare our results with the response to bacterial endotoxins, where a similar pattern is found Thus *Greene et al* have demonstrated a dosage response relationship over a certain range in the response to treatment with Piromen in thermally injured rats (10) A similar pattern is found when the migration of leukocytes after intraperitoneal injection of endotoxin in mice is estimated (11) Since the materials are not pyrogenic in rabbit in 5 mg dose (2) the effect of our material from blood is hardly due to contaminating pyrogens

The mechanism which leads to the death of the animal during cold stress is probably complex *Selye* (12) has pointed out that cold stress represents an ideal example of alarm reaction with haemoconcentration, hypoglycaemia and intestinal haemorrhages, reactions which are not obscured by reactions due to the shockproducing stimulant itself The experiments reported here were not carried out to investigate the mechanism of cold stress, and no detailed examination of our animals

1 Unpublished results

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ETHYLENE OXIDE STERILIZATION WITHOUT SPECIAL EQUIPMENT[1]

By

KERSTIN WINGE HEDÉN

Received 11 xii 62

In the prevention of nosocomial infections, problems of sterilizing hospital equipment are important Most types of endoscopes, often with delicate and expensive optics, have long presented difficulties as regards sterilization More and more refined instruments with complicated electrical parts are coming into use in hospitals, as are many varieties of plastics (*Engley* 1951, *Grundy, Rdzok, Remo, Sagen & Sylvester* 1957) High temperatures and steam usually have deleterious effects on such instruments and materials Satisfactory sterilization is, however, necessary and this is why gases such as ethylene oxide have increasingly been used as sterilizing agents in the past decade (*Bekker & Onvlee* 1956, *Davis, Wolkoff & Leonards* 1957, *Diding & Kjellman* 1958, *Skeehan* 1959, *Thomas* 1960, *Bracken, Wilton-Davies, Weale & Kelsey* 1960, *Kelsey* 1961) Since the potentialities of gas sterilization nevertheless are not fully utilized in many sectors of hospital routine, a review of the general properties and uses of ethylene oxide would seem to be warranted

General Properties

[1] ... Swedish National Association against Heart and Chest Diseases

11 *Ecklin B* Auslosung einer lokalen Leukozytenreaktion durch Bakterien und Bakterienprodukte und ihre Beeinflussbarkeit durch Lipopolysaccharide Arch int pharmacodyn *127* 190 1960
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14 *Lillehei R C* *Longerbeam J K* & *Rosenberg J C* The Nature of Irreversible Shock its Relationship to Intestinal Changes Shock Pathogenesis and Therapy An International Symposium Springer Verlag Berlin Gottingen Heidelberg 106 1962
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centrations over fairly long periods can produce nausea vomiting headache and dizziness (*Blackwood & Erskine* 1938) Pulmonary oedema may follow exposure to high concentrations of the gas Gaseous ethylene oxide in a concentration of about 6 mg per liter can be tolerated for an inhalation period of at most 60 minutes

Liquid ethylene oxide as a rule evaporates from the skin so rapidly that cooling is the only result More protracted contact between the skin and substances in which the gas has been dissolved (rubber leather plastic etc) may give rise to blistering, however (*Royce & Moore* 1955) This has been observed when for instance, laboratory staff used rubber shoes which shortly before had been sterilized with ethylene oxide (*Phillips* 1958) Sensitization to ethylene oxide has been experimentally evoked (*Sexton & Henson* 1949 and 1950) Hypersensitivity reactions, however are rare

Practical Use

Sterilization with ethylene oxide is dependent upon the following factors exposure time, concentration (partial pressure of ethylene oxide in the chamber), temperature and humidity In most cases the recommended concentration of ethylene oxide is 450 mg per liter for 5 hours at a temperature of about 55° C If the concentration can be raised to 1000 mg per liter, the exposure time can be reduced to c 3 hours In order to reach such concentrations, the exposure must take place at pressures of 2 to 6 kg per cm² when 10 per cent ethylene oxide in CO_2 is used and at 1 to 2 kg per cm² when 20 per cent ethylene oxide is used For production of these conditions and the necessary temperature and humidity, special apparatus has been employed as a rule Several autoclave like designs are now on the market, for instance American (American Sterilizer Co Carboxide , Cryoxide-, Oxyfume equipment) German (DMB Sterivit equipment) and Finnish (Oy Santasalo-Sohlberg Freoxide equipment)

By using a mixture of 11 per cent ethylene oxide and 89 per cent fluorinated hydrocarbons (freon) it has been found possible to effect sterilization at normal atmospheric pressure Thus, *Schley, Hoffman & Phillips* (1960) published a method for sterilizing clothes, etc by placing them in polythene bags together with a container of ethylene oxide-freon The container was punctured and the gas escaped into the bag, where it gave a concentration of about 500 mg per liter Like many other plastics however, the bags used in these experiments were permeable to the gas (*Waack, Alex Frisch & Seware* 1955) and, as a result, exposure times and concentrations could hardly be reliably controlled

The aims of the present study, based on the desirability of a simple gas sterilization method suitable for routine use, were as follows

1 To find a material suitable for making sterilizing bags and having the following properties

these substances An important point is that *ethylene oxide is active even in the presence of large amounts of organic substances* It also penetrates through shallow *layers of oil or water*, which are thereby sterilized Ethylene oxide rapidly dis appears after use, but *relatively long airing* is advisable after sterilization of thick or dense materials, for instance rubber or plastic, in which it may have dissolved (*Phillips & Warshowsky* 1958)

In water ethylene oxide is hydrolyzed to ethylene glycol, which has considerably *lower sterilizing potency* At room temperature this reaction takes place slowly but it is accelerated by various catalysts, particularly mineral acids and some metals such as copper

TABLE 1

Ethylene Oxide Mixtures for Sterilization

Commercial name	Maker	Ethylene oxide (%)	Inert gas (%)	Properties	Sterilization pressure
T-gas	Deut Gesellsch f Schadlingsbekampf mbH Frankfurt a/M	90	10 CO_2	explosive	
Etox	London Fumigation Co Ltd London				
Oxyfume	Union Carbide & Carbon Corp New York 17 N Y	20	80 CO_2	explosive	1 atm
Ato	*Sterisit GmbH* Mainz a/Rh	*12–15*	*88–85 CO_*	*nonexplosive*	*4–7 atm*
Cartox	Deut Gesellsch f Schadlingsbekampf mbH Frankfurt a/M	10	90 CO_2	nonexplosive	1 2 9 atm
Carboxide	Union Carbide & Carbon Corp New York 17 N Y	10	90 CO_2	nonexplosive	1 2 9 atm
Cryoxide	American Sterilizer Co *Erie Penn USA*	11	44 5 CCl_2F_2 44 5 CCl_3F	nonexplosive	1 35 2 2 atm
Freoxid	Oy Santasalo Sohlberg Helsinki	10	90 Freon	nonexplosive	1 2 8 atm

Ethylene oxide is highly explosive in air even in concentrations as low as 3 per cent (*Hess & Tilton* 1950) For sterilization in hospitals therefore nonexplosive and nonflammable mixtures are usually employed (*Haenni Affens Lento Yeomans & Fulton* 1959) Table 1 presents data concerning some mixtures It is based largely on a report by *Bruhin Bühlmann Vischer & Lammers* (1961) Fluorinated hydro carbons ethylene oxide mixtures are rapidly effective even at ordinary atmospheric pressure As compared with corresponding mixtures of ethylene oxide and carbon dioxide they have lower vapour pressure and contain more ethylene oxide per unit of volume

(*Waller & Greeson* 1932 *Amdur & Mead*
ter & Spencer 1956 *Jacobson Hackley &*
ide vapour irritates the respiratory tract in much the same way as ammonia itching and burning sensations may occur in the eyes The gas is more toxic to small than to large animals Inhalation of low con



Fig 1.

filled After some practice this can be reduced to a single extra spraying The bag can thereafter be stored at room temperature. The bags should be opened on a balcony or by an open window or under a hood, since the gas has some toxic properties

Liquid ethylene oxide can cause changes in the shape of or cracks in some plastics (*Spencer & Bahnson* 1958). It is therefore important that the outflow of the small tins that are placed in sterilizing bags should not be directed towards delicate material The mouthpiece can for instance be covered with cottonwool or gauze in which the liquid can vapourize

B Sterilization in Commercial Ethylene Oxide Equipments

Two commercial ethylene oxide sterilizers have been tested The makers recommend a sterilization temperature of 55° C, but with both types of apparatus other temperatures can be used

One apparatus (DMB-Sterivit) was run at a pressure of 6 4 kg per cm² and the recommend sterilization time was 1 hour (*Gewalt & Fischer* 1959). A mixture of 15 per cent ethylene oxide and 85 per cent CO_2 was used. Humidity was obtained with the aid of a piece of plastic sponge which was moistened befor[illegible]

The other a[illegible] pressure of ab[illegible] and 90 per ce[illegible] intake of a small amount (1 ml) of water after a vacuum had been created With regard to sterilization times, the makers provided the following table:

Pressure (kg cm²)	*Concentration of ethylene oxide (mg liter)*	*Exposure time (hours)* 40° C	55° C
0 5	600	4–6	3–5
1 0	800	3–5	2–4
1 5	1000	2–4	1½–3
2 0	1200	1½–3	1–2

a) impermeability to ethylene oxide,
b) strength, and
c) ease of sealing

2 To study the efficiency of this system

3 To compare it with conventional ethylene oxide sterilizers and with a standard hospital autoclave converted by simple measures into an ethylene oxide sterilizer

A brief preliminary report has been published in the form of an abstract (*Winge-Heden* 1962)

METHODS

Three methods of ethylene oxide sterilization were used

A In three foil bags
B In commercial ethylene oxide sterilizers
C In a converted hospital autoclave

A Sterilization in Three Foil Bags

Material Since the majority of plastics are more or less permeable to ethylene oxide plastic alone was not considered suitable for making sterilizing bags A material—"three foil"—made by Akerlund & Rausing[1] was found to be suitable however and was kindly made available to me The foil was supplied in rolls of varying widths As its name implies it has three layers The outermost layer is of fairly strong but flexible paper Beneath this is a layer of aluminium foil and innermost a thin layer of polythene plastic The three layers are closely adherent to each other and can only be separated with difficulty This renders the material tough and strong It is highly resistant to handling and moisture and the paper surface is suitable for notations The aluminium foil makes the material gas impermeable Because of the plastic lining the bags are extremely simple to make since all that is required is to place two plastic layers in apposition and then run over the material with a hot household iron A fairly broad seam can thus be obtained and through this thin and broad plastic barrier no significant diffusion of gas occurs as indicated by the observation that the gas remained in bags stored as long as 4 months

sterilization in these bags

The ethylene oxide freon

n be released by a valve or by puncture of the tin In my experiments the gas was supplied by a Finnish firm (Oy Santasalo Sohlberg) Three sizes of tin are available (containing 0 1 0 4 and 0 6 kg gas mixture *i e* 10 per cent ethylene oxide and 90 per cent freon) The gas in the tins is under pressure and is sprayed out through a small valve which can be manipulated from outside the bag

Making three foil sterilizing bags and technique of sterilization For each bag about 60 × 100 cm of three foil were used The material was folded double with the plastic surfaces against each other Three sides of the bag were then sealed with the aid of a hot iron The bacteriological test pack (see below) an 0 1 kg tin of freon ethylene oxide and a small moistened swab of cottonwool or a moist piece of filter paper were placed in the bag as shown in Fig 1 As much as possible of the air in the bag was pressed out before the fourth side was sealed by ironing In this con

ticles to be sterilized preferably should not

be forced out For such articles larger bags

used The gas is released without difficulty

felt from outside the bag Spraying is interrupted after a few seconds while the operator observes how much the bag expands when the liquid is vapourized Spraying and pause are repeated until the bag is

1 Akerlund & Rausing Ltd Lund Sweden Paper 70 g m² + Al 0 09 mm + polyeten 45 g/m Total weight 140 g/m²

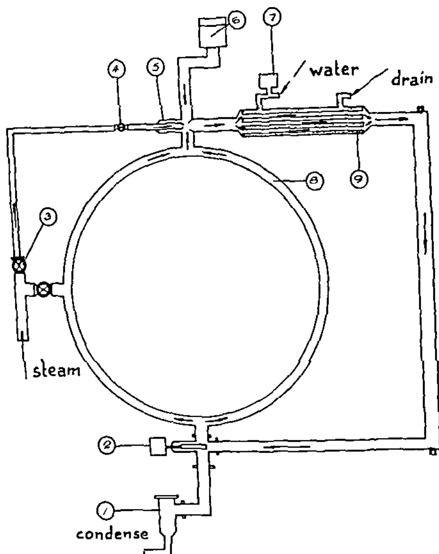


Fig 2

1 Condensate trap
2 Sensing element for temperature
3 Valve
4 Fine adjustment valve
5 Ejector
6 One way air valve opened by vacuum in jacket
7 Magnetic valve
8 Steam jacket
9 Cold water cooler

valve which hitherto had been in [illegible] et to 2 until the pressure in [illegible] he door screws were further [illegible] setting the central valve to [illegible] k only a few seconds After

C Sterilization in a Converted Steam Autoclave

At the research department of the Clinic for Thoracic Surgery an ordinary horizontal hospital autoclave (Getinge, type SAL 8), jacketed and provided with a central valve, volume c. 500 liters, was modified so that it could be used alternatively for ethylene oxide or steam Such modifications have been tested by other workers (*Kaye* 1950, *Skeehan, King & Kaye* 1956, *Spencer & Bahnson* 1958, *Sager & Iten* 1959 *Schley, Hoffman & Phillips* 1960, *Freeman & Barwell* 1960) The arrangement here selected, however, was found to be simple and cheap as well as extremely reliable For these reasons some details of the construction of the apparatus, worked out in collaboration with Getingeverken Ltd, are now presented

The central valve of the autoclave controls the following connection (O = open C = closed):

a air intake—autoclave chamber,
b. steam from mantle to chamber,
c. steam from chamber to exhaust, and
d autoclave chamber to vacuum pump

The valve has 6 positions:

1. *Stop, preheating* (a/O, b/C, c/C, d/C).
2 *Vacuum* 65 cm Hg (a/C, b/C, c/C, d/O),
3. *Pressure rise* 2 0–2 5 atmospheres (a/C, b/O, c/C, d/C).
4 *Sterilization* (a/C, b/O, c/O, d/C).
5. *Steam removal* (a/C, b/C, c/C, d/O),
6 *Drying* (a/O, b/C, c/C, d/O).

The air intake of the autoclave is fitted with a small filter. In the conversion to an alternative ethylene oxide sterilizer, a threeway cock was inserted between the autoclave and the filter. By means of this cock the autoclave chamber or, more correctly the central valve could be connected with the filter or, *via* a reinforced tube with a "Cartox" cylinder which was provided with a pressure-reducing valve. The filled cylinder weighed approximately 85 kg It contained about 25 kg of gas mixture and was suspended in a spring balance (Salter No 235, can weigh 100 kg with an accuracy of 0 5 kg) This arrangement was necessitated by the inadvisability of using more than about 80 per cent of the gas mixture, since otherwise there is a risk that the concentration of CO_2 will become excessive

An expanding gaseous mixture chills rapidly, and therefore it is desirable that the pressure-reducing valve, which is left open throughout the sterilizing phase, should be of modern heatable type In addition, the communication between the central valve and the autoclave chamber must partly be steam jacketed as a protection against condensation in bends and couplings It must also be provided with a safety valve adjusted to 5 kg per cm² and connected to the steam blow off pipe of the apparatus Another safety valve, set at 3 kg per cm², can open the tube between the central valve and the autoclave chamber to the steam blow off pipe when the pressure exceeds that level Othewise no changes are required apart from those for thermoregulation The water ring pump for production of vacuum may be used for instance, since the gas is highly soluble in water The pump however, should be placed in a relatively well-ventilated site

A simple thermoregulating system, which does not interfere with the normal steam sterilizer function was designed as shown in Fig 2 The thermostat (2) directs the flow of cold water through the magnetic valve (7), so that the steam sprayed through the ejector (5) is cooled A valve (6) draws in a fixed volume of air which is mixed with the steam leaving the jacket (8) of the autoclave To the intake of the jacket at (2) flows a mixture of steam air and condensate which maintains the desired temperature of the thermostat The condensate is removed through a trap (1) and the air and steam mixture flows through the jacket and heats the autoclave

The procedure for ethylene oxide sterilization with this apparatus was as follows The main steam valve and the valves to the gas heater and the jacket thermoregulation (No 3 in Fig 2) were opened The electrical thermostat, set at +60° C, was activated The nonsterile material was placed in the apparatus and the door was carefully closed The gas cylinder was lifted up in the spring balance, its weight was noted and the balance again unloaded The threeway cock at the air filter was closed and also the escape valve for condensate from the autoclave chamber The central

ged centrifuge tubes, in polythene foil bags (0 05–0 08 mm) or in nylon foil bags (0 05 mm thick)

In the sterilizers in which release of the gas was proceded by a vacuum phase, it was necessary to tie a single layer of gauze over open tubes and dishes to prevent the light test foil from being whirled up The same procedure was also used for sterilization in three foil bags, since otherwise there was risk that the test slips might fall out of the dishes when the bags were handled

%, sterile samples (50 — 100)	Number of samples	Extra humidity source	Enclosure of samples	Time in hrs	Temp °C
	33 26 27	One water drop on each sample	Open petridishes	6	22
	32 30 36	One water drop on each sample	Nylon bags	6	22
	20	Moist cotton swab in the nylon bags	Nylon bags	6	22
	3 13 13	—	Nylon bags	6	22
	18 20 19	Moist cotton swab in the nylon bags	Nylon bags	6	37
	67 20 17	One water drop on each sample	Nylon bags	18	22
	10 5 10	One water drop on each sample	Open test tubes length about 10 cm	18	22
	63 24 19	Moist cotton swab in the nylon bags	Nylon bags	18	22
	13 8 13	—	Nylon bags	18	22
	22 37 36	—	Petridishes with lid	18	22

Fig 3
Three foil bags
Samples Aluminiumfoils charged with

——— spores of B brevis
– – – Staph aureus
Str faecalis

this the valve was adjusted to 1 and the sterilizing gas was turned on the pressure reducing valve set to 2 atmospheres *Six hours after this pressure had been reached* which took about 15 minutes, the flow of gas was turned off and the material was aired by setting the central valve to 6 When the pressure had fallen to atmospheric level, the *aircock was opened About 15 minutes later the central valve was again* turned to 1 and the initially described steps were repeated in reverse

The gas consumption per run was 2 to 3 kg Small leaks in valves and elsewhere *produced minor variations in this respect Since the gas also diffuses out through* rubber gaskets etc the cylinder should be turned on throughout the time of sterilization

Bacteriological Investigation Technique

The efficiency of all the described methods was tested by determination of the bactericidal potency with respect to

1) spores (of *Bacillus brevis*), and

2) vegetative bacteria (*Staphylococcus aureus* 209 Oxford, and a local *strain of Streptococcus faecalis*)

The *Bacillus brevis* strain was obtained from the bacteriological department of Karolinska Institute, where it was used for a simplified autoclave test (*Heden & Markula* 1962) requiring micro-organisms with *high heat resistance*

Suspensions of these micro-organisms were carefully dried on punched-out rounds of filter paper 10 mm in diameter (*Schleicher & Schüll* no 2208) or on 0 015 mm thick squares of aluminiumfoil Micro organisms dried on metal foil present a considerably more exacting test of ethylene oxide sterilization than do micro-organisms on porous material When this difference had been confirmed in all the tested types of apparatus, therefore, I ceased to use filter paper and confined the tests to the pieces of aluminium foil

TABLE 2

Data Concerning the Tested Sterilization Methods

Type of apparatus	Gas mixture		Time hours	Temperature °C	Pressure kg cm²
	ethylene oxide %	inert gas %			
Commercial high pressure sterilizer	15	85	1	55	6 4
Commercial low pressure sterilizer	10	90	4 6	55	2 4
Converted autoclave	10	90	6	60	2 0
Sterilizing bags	10	90	6 18	22	1 0

Wrapping Technique

In order to study the influence of wrapping the articles on the results of ethylene oxide sterilization, the placement of the test foil was varied, for instance in open or closed Petri dishes, in open and cottonwool-plug-

% Sterile samples (0 – 50 – 100)	Number of samples	Extra humidity source	Enclosure of samples	Time in hrs	Temp °C
	20, 20	One water drop on each sample	Open petridishes	6	55
	20, 24			4	55
	15, 15, 15	—		4	55
	10, 10, 10	—	Open test tubes horizontal	6	55
	5, 5, 5	—	Petridishes with lid	4	55
	10, 5, 5	Moist cotton swab in the polyethylene bags	Polyethylene bags	4	55
	15, 14, 15			6	55
	5, 5, 5	Moist cotton swab in the nylon bags	Nylon bags	16	55
	9, 9, 9			6	55
	3, 3, 2			4	55

Fig 5

Low pressure ethyleneoxide sterilizer
Samples Aluminiumfoils charged with

——— spores of B brevis
— — — Staph aureus
Str faecalis

Variations in sterilization time, temperature, pressure and gas mixture in the different experiments are shown in Table 2

RESULTS

The results of the experiments are presented in Figures 3–8 A few bizarre results occurred For instance, the effect was sometimes better after short than after longer exposure to sterilizing gas, but most of these deviations occurred with the commercial low pressure apparatus

Humidity can be of decisive importance in ethylene oxide sterilization (*Phillips & Kaye* 1949). Tests were therefore made with dry aluminium squares and with squares which had been moistened with a small drop of water. When dry pieces of foil were packed in nylon or polythene bags, a moist swab was as a rule also inserted, but in other tests the foils were packed dry. In studies of the commercial sterilizers, the maker's recommendations with regard to humidity were always followed.

% Sterile samples	Number of samples	Extra humidity source	Enclosure of samples	Time in hrs	Temp °C
	Samples: Aluminiumfoils				
0 50 100					
	14	One water drop on each sample	*Open petridishes*	1	55
	20				
	18				
	45	—	*Open petridishes*	1	55
	20				
	20				
	38	—	Open test tubes horizontal	1	55
	10				
	10				
	20	—	Petridishes with lid	1	55
	20				
	20				
	15	Moist cotton swab in the polyethylene bags	*Polyethylene bags*	1	55
	17				
	13				
	20	Moist cotton swab in the nylon bags	Nylon bags	1	55
	19				
	20				
	20	—	Nylon bags	1	55
	5				
	5				
	Samples: Filterpaperdiscs				
	22	—	*Open petridishes*	1	55
	23				
	26				
	38	—	Open test tubes vertikal	1	55
	38				
	38				
	20	—	Nylon bags	1	55
	20				
	20				

Fig. 4.

High pressure ethyleneoxide sterilizer.
Samples: *Test slips charged with*

——— spores of B. brevis
— — — Staph. aureus
Str. faecalis

% Sterile samples (0 – 50 – 100)	Number of samples	Extra humidity source	Enclosure of samples	Time in hrs	Temp °C
	20, 20, 20	One water drop on each sample	Open petridishes	6	55
	24	One water drop on each sample	Open petridishes	4	55
	15, 15, 15	—	Open petridishes	4	55
	10, 10, 10	—	Open test tubes horizontal	6	55
	5, 5, 5	—	Petridishes with lid	4	55
	10, 5, 5	Moist cotton swab in the polyethylene bags	Polyethylene bags	4	55
	15, 14, 15	Moist cotton swab in the polyethylene bags	Polyethylene bags	6	55
	5, 5, 5	Moist cotton swab in the nylon bags	Nylon bags	16	55
	9, 9, 9	Moist cotton swab in the nylon bags	Nylon bags	6	55
	3, 3, 2	Moist cotton swab in the nylon bags	Nylon bags	4	55

Fig 5

Low pressure ethyleneoxide sterilizer
Samples Aluminiumfoils charged with

——— spores of B brevis.
– – – – Staph aureus
Str faecalis

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I shall return to them in the discussion From the results, some general conclusions could be drawn

* Sterile samples (0 — 50 — 100)	Number of samples	Extra humidity source	Enclosure of samples	Time in hrs	Temp °C
	15, 15, 15	One water drop on each sample	Open petridishes	6	60
	20, 20, 20	One water drop on each sample	Nylon bags	6	60
	15, 15, 15	—	Open petridishes	6	60
	28, 25, 30	—	Open test tubes length about 10 cm horizontal	6	60
	10, 10, 10	—	Open test tubes vertikal	6	60
	18, 19, 20	—	Petridishes *with lid*	6	60
	25, 24, 25	Moist cotton swab in the nylon bags	Nylon bags	6	60
	13, 10, 10	—	Nylon bags	6	60

Fig 6
Modified steam sterilizer
Samples Aluminiumfoils charged with

——— spores of B brevis
– – – Staph aureus
- - Str faecalis

One hundred per cent sterility was obtained with all methods (excluding the low-pressure apparatus) if the test slips
1) were kept in open dishes during sterilization, and
2) were moistened prior to sterilization

DISCUSSION

As Figures 3 to 8 show, wide variations were seen in the results The factors, apart from temperature, time and pressure, which influenced sterilizing efficiency were

% Sterile samples (0 50 100)	Number of samples	Extra humidity source	Enclosure of samples	Time in hrs	Temp °C	MODIFIED STEAM STERILIZER
	8	Moist cotton swab in the polyethylene bags	Bottom of cottonplugged horizontal testtubes in polyethylene bags	6	60	Samples Aluminiumfoils charged with
Spores	8	—	Bottom of cottonplugged horizontal testtubes in polyethylene bags	6	60	—— Spores of B brevis – – Staph aureus
	21 21	Moist cotton swab in the polyethylene bags	Bottom of cottonplugged horizontal testtubes in polyethylene bags	6	60	Samples Filterpaperdiscs charged with
	21 21	—	Bottom of cottonplugged horizontal testtubes in polyethylene bags	6	60	—— Spores of B brevis – – Staph aureus
						HIGH PRESSURE ETHYLENEOXIDE STERILIZER
	31 30	—	Bottom of cottonplugged horizontal testtubes in polyethylene bags	1	55	Samples Filterpaperdiscs charged with
	29 31	Moist cotton swab in the polyethylene bags	Bottom of cottonplugged horizontal testtubes in polyethylene bags	1	55	—— Spores of B brevis – – Staph aureus

Fig 7

I shall return to them in the discussion From the results, some general conclusions could be drawn

% Sterile samples (0 50 100)	Number of samples	Extra humidity source	Enclosure of samples	Time in hrs	Temp °C
———	15	One water drop on each sample	Open petridishes	6	60
— — —	15				
- - -	15				
———	20		Nylon bags	6	60
— — —	20				
- - -	20				
———	15	——	Open petridishes	6	60
— — —	15				
- - -	15				
———	28	——	Open test tubes length about 10 cm horizontal	6	60
— — —	25				
- - -	30				
———	10	——	Open test tubes vertikal	6	60
— — —	10				
- - -	10				
———	18	——	Petridishes with lid	6	60
— — —	19				
- - -	20				
———	25	Moist cotton swab in the nylon bags	Nylon bags	6	60
— — —	24				
- - -	25				
———	13	——	Nylon bags	6	60
— — —	10				
- - -	10				

Fig 6

Modified steam sterilizer
Samples Aluminiumfoils charged with

——— spores of B brevis
— — — *Staph aureus*
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DISCUSSION

As Figures 3 to 8 show, wide variations were seen in the results The factors, apart from temperature, time and pressure, which influenced sterilizing efficiency were

aluminium foil was used as test material Table 3 illustrates the difficulty of sterilizing aluminium foil as compared with filter paper The 45 spore bearing aluminium foil slips and the 25 spore bearing filter paper discs were exposed to ethylene oxide for one hour at 55° C Of the former only 6 were rendered sterile whereas all the filter paper discs were sterilized

Humidity

The crucial importance of moisture for ethylene oxide sterilization has already been mentioned *Phillips* (1949) stated the optimal relative humidity to be about 30 per cent In a more recent report (1961) he showed that desiccated spores and vegetative bacteria can become totally resistant to ethylene oxide This phenomenon does not affect all the microbes in a suspension but only a minority In the technique which I used even a very small number of surviving micro organisms show up Phillips further reported that after direct moistening with water the resistant bacteria reassumed their usual sensitivity Attempts to condition the desiccated microbes in high relative humidity showed that they must be left in the moist atmosphere for several days (4 days at 98 per cent relative humidity and 6 days at 78 per cent) before normal sensitivity to ethylene oxide was regained

The possibility that a very few of the test micro organisms were so intensively dried that they became resistant to ethylene oxide presumably explains some of the apparently paradoxical results in my experiments The test slips often were prepared in fairly large batches which were gradually used up This meant that in some experiments the slips had been dried for only 24 hours but in other experiments the slips could have been stored dry for 2 to 3 weeks before use The atmospheric humidity in the laboratory was very low (c 13 to 15 per cent) for long periods (measured with the aid of Phillips Air Humidity indicator PR 9650)

Figure 8 shows that freshly prepared test slips were appreciably easier to sterilize than slips which had been stored dry for some length of time

When the test slips were placed in plastic foil bags simultaneous insertion of a moist cottonwoll swab considerably improved the results But the moist swab clearly was not always adequate if the slips were very dry The time during which the very dry slips lay in the humid atmosphere was then probably too short Such an effect was specially noticeable with the high pressure sterilizer which used a very short sterilization time Considerably better results were also obtained in the experiments with three foil bags in which the longest sterilization times were used But here too 100 per cent sterility might not result when very dry slips were tested

From the purely practical point of view these observations cannot be

a) the nature of the nonsterile test material (foil or paper),
b) humidity, and
c) wrapping of the test material

% Sterile samples	Number of samples	Extra humidity source	Enclosure of samples	Time in hrs	Temp °C
0 50 100					
———————	10		Nylon bags		
— — — — —	10	——		24	22*
- - - - - - -	10				
	6				
	6	——		24	37§
	6				

Fig 8
Three foil bags
Samples Aluminiumfoils charged with
——— spores of B brevis
— — — Staph aureus
- - - - - Str faecalis

* *Samples prepared the day before test*
§ Samples prepared two months before test Low Humidity during storage

TABLE 3
Comparison of Sterilization Results on Spore-Bearing Test Materials

Type of test slip	No of test slips		% sterile
	sterile	tested	
Aluminium foil	6	45	15
Filter paper	25	25	100

Sterilized in open Petridishes for 1 hr Temp 55° C
Pressure 6 4 atm No extra humidity

Nature of the Nonsterile Test Material

As mentioned, pieces of aluminium foil with dried micro-organisms presented a much more exacting test of sterilizing efficiency than did porous paper The main reason for this higher resistance to ethylene oxide would seem to be the impermeability of aluminium foil to the gas, in constrast to filter paper During desiccation, moreover, a protective film of salt crystals forms around the micro-organisms (*Mathews & Hofstad* 1953, *Phillips & Kaye* 1949)

Many of the instruments which can with advantage be sterilized by ethylene oxide are made of polished metal or glass, both of course impermeable to the gas In the later part of the study, therefore, only

is opened As mentioned extraction of sterilized articles should be done either in the open air or under a closed hood so as to avoid inhalation of the escaping gas

Bulky objects containing large volumes of air that cannot be manually forced out are not suitable for sterilization in three foil bags of the normal size since the trapped air will render the ethylene oxide concentration inadequate For such articles gas sterilization should be preceded by a vacuum phase

Sterilization in three foil bags is an extremely simple method which gives good results without any special apparatus The cost of a bag which holds about 12 liters is approximately 1 Sw crown The gas for this operation costs about 6 Sw crowns

The two commercial sterilizers gave widely varying results With the low pressure apparatus the variation was too great to permit reliable assessment With the high pressure apparatus 100 per cent sterility was obtained when the test slips of aluminium lay in open dishes and were moistened with a drop of water before sterilization The tests with filter paper discs gave considerably better results Metal or glas articles thus should not be placed in closed boxes or wrapped in foil *before sterilization* in such commercial apparatus Nor should dipping of the instruments in water be omitted before sterilization The brevity of the exposure periods probably explains the comparatively poor results with wrapped test slips In brief ethylene oxide exposure the gas does not have time to diffuse through foil wrappings or to become distributed in pockets of air that can exist in tubes or dishes When closed Petri dishes are used the high pressure may compress the lid against the edge of the dish thus delaying diffusion of the gas Brief exposure further implies that desiccated micro organisms are not adequately moistened even [illegible]

Exten [illegible]
result [illegible]
most [illegible]
gave [illegible]

The autoclave converted for ethylene oxide sterilization like the high pressure commercial sterilizers gave satisfactory results in tests using aluminium foil slips which had been moistened with a drop of water and were exposed in open dishes In addition test slips wrapped in nylon foil together with a moist cottonwool swab were sterilized in all but one test The preliminary vacuum phase and the longer exposure to ethylene oxide undoubtedly explain the superior results as compared with those from the high pressure commercial autoclave The importance of the vacuum phase was also demonstrated by the experiments in which the test slip was placed in a cotton plugged test tube and then in a bag of polythene foil With this arrangement the converted autoclave was clearly superior to the high pressure commercial autoclave A pertinent point is that bags containing bulky articles should not be

ignored Materials which are to be sterilized after very dry storage for some length of time thus should preferably be dipped in water prior to ethylene oxide sterilization, or should be packed together with a source of moisture

By comparison with other bacteriological studies of the sterilizing action of ethylene oxide, some of my experiments may seem to have given poorer results But scrutiny of other writers' reports shows that in many cases their test material was dried only for an hour or two and sometimes not at all, which appreciably facilitated sterilization In all my experiments the test slips had been dried for at least 24 hours and often for appreciably longer periods Since they therefore constituted a very severe test, any good results must be considered to have a wide margin of safety which, of course, is desirable in all sterilizing of hospital material

Wrapping of the Nonsterile Material

It was to be expected that the best results of ethylene oxide sterilization should be obtained when the test material lay open In hospital work, however, wrapping of the various articles, preferably in bacteria proof foil, is frequently necessary for ease of handling, transportation and storage and to minimize risk of contamination Nylon film, polyvinyl chloride and polythene are widely used as wrappings for both steam and ethylene oxide sterilization

In my experiments nylon foil was found to be a highly suitable wrapping, but polythene foil also gave good results In the high-pressure apparatus the latter gave better results, but detailed comparisons of the various plastic foils were not made

If it is desired to sterilize articles in boxes or trays, these should be left open and sterilization should preferably be done in an apparatus which permits a preliminary vacuum phase

Sterilization in three-foil bags gave results which were equivalent to and often better than those where commercial sterilizers or the converted autoclave were used The superior effect probably was attributable to the longer sterilization times With the three-foil method, for instance, dry aluminium foil slips in closed dishes were rendered sterile, which could not always be achieved by the other methods The recommendations for wrapping of the nonsterile articles must be observed, however, which implies that if these articles are packed in nylon, etc a moist cottonwool swab or filter paper must be inserted under the wrapping The articles can also be placed directly in the three-foil bag, when it is important not to omit the moist swab in the bag

Nylon foil was found to give better results than polythene foil in sterilization at low pressures As a general rule, the articles to be sterilized should be wrapped in nylon before being placed in three-foil bags, since this considerably facilitates sterile handling when the bag

is opened As mentioned extraction of sterilized articles should be done either in the open air or under a closed hood so as to avoid inhalation of the escaping gas

Bulky objects containing large volumes of air that cannot be manually forced out are not suitable for sterilization in three foil bags of the normal size since the trapped air will render the ethylene oxide concentration inadequate For such articles gas sterilization should be preceded by a vacuum phase

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Extension of the exposure [illegible]
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completely sealed before sterilization, in order to avoid the danger of bursting of the foil during the vacuum phase A small slit should be left open and can easily be sealed with tape immediately after sterilization

Sterilized articles of rubber, plastic or other material in which ethylene oxide has become dissolved must be aired before use (*Phillips & Kaye* 1949, *Grundy, Rdzok, Remo, Sagen & Sylvester* 1957, *Freeman & Barwell* 1960) This is particular important as regards articles which come into direct contact with respiratory passages, blood vessels or other tissues (Ethylene oxide has also been successfully used for sterilizing various types of tissue grafts) In the converted autoclave a vacuum can be created also after sterilization, which considerably facilitates rapid removal of ethylene oxide With the other methods airing at room temperature is necessary The time of airing is dependent upon the density and nature of the sterilized articles

As regards cost of ethylene oxide sterilization in the converted autoclave, the price of the "Cartox" gas is approximately 240 Sw crowns per 25 kg, which implies that the cost of a run is about 30 crowns In these experiments about 2 kg of gas were used per run It is recommended that not more than four-fifths of the gas in the tube should be used, since the concentration of ethylene oxide in the last portion of the mixture may fall below the figure stated on the tube The volume of the sterilization chamber is 500 liters and therefore large numbers of articles can be sterilized at one time

SUMMARY

Three methods for ethylene oxide sterilization are described

1) In "three foil" bags,
2) in commercial sterilizers, and
3) in a converted steam autoclave

1 The three-foil bag method can be used for ethylene oxide sterilization when special apparatus is not available The foil is composed of paper, aluminium and plastic It is impermeable to ethylene oxide and with the aid of an ordinary household iron, can easily be made into bags of desired size The nonsterile material, which may be wrapped in nylon film is placed in the three foil bag together with a small ethylene oxide freon aerosol can, the valve of which can be manipulated from outside the bag Instruments can preferably be dipped in water before being wrapped in plastic foils or can be placed directly in the three-foil bag, along with a source of moisture If the articles have been stored under extremely dry conditions before sterilization, the direct moistening is essential Exposure to ethylene oxide in three-foil bags for 6 hours at room temperature gives full sterility

2 In a commercial ethylene oxide sterilizer which functions with a pressure of 6 4 kg per cm^2, the same bacteriologic studies were made

as with the three foil bags The results from the former method were less good undoubtedly because of the shorter period of exposure to the gas Full sterility was nevertheless obtained when the test slips were treated openly in the sterilizing chamber and had previously been moistened with water Wrapping in plastic of the articles to be sterilized is not recommended when this apparatus is used The method is the quickest of those tested the sterilizing time being one hour

3 A 500 liter autoclave was modified so that it could be used for both heat and gas sterilization When the temperature was 60° C and the chamber was filled with ethylene oxidcarbon dioxide up to a pressure of 2 atmospheres, full sterility was obtained after 6 hours of all test material in open dishes and in nylon bags provided that it had been directly moistened with water before sterilization Even test material wrapped in nylon foil together with a moist swab was sterilized in practically all cases This converted autoclave is particularly suitable when large numbers of articles or bulky objects are to be sterilized

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Three methods for ethylene oxide sterilization are described:

1) In "three-foil" bags,
2) in commercial sterilizers, and
3) in a converted steam autoclave.

1. The three-foil bag method can be used for ethylene oxide sterilization when special apparatus is not available. The foil is composed of paper, aluminium and plastic. It is impermeable to ethylene oxide and with the aid of an ordinary household iron, can easily be made into bags of desired size. The nonsterile material, which may be wrapped in nylon film, is placed in the three-foil bag together with a small ethylene oxide/freon aerosol can, the valve of which can be manipulated from outside the bag. Instruments can preferably be dipped in water before being wrapped in plastic foils, or can be placed directly in the three foil bag along with a source of moisture. If the articles have been stored under extremely dry conditions before sterilization, the direct moistening is essential. Exposure to ethylene oxide in three foil bags for 6 hours at room temperature gives full sterility.

2. In a commercial ethylene oxide sterilizer which functions with a pressure of 6.4 kg per cm², the same bacteriologic studies were made

as with the three-foil bags The results from the former method were less good, undoubtedly because of the shorter period of exposure to the gas Full sterility was nevertheless obtained when the test slips were treated openly in the sterilizing chamber and had previously been moistened with water Wrapping in plastic of the articles to be sterilized is not recommended when this apparatus is used The method is the quickest of those tested, the sterilizing time being one hour

3 A 500 liter autoclave was modified so that it could be used for both heat and gas sterilization When the temperature was 60° C and the chamber was filled with ethylene ox de arbon dioxide up to a pressure of 2 atmospheres, full sterility was obtained after 6 hours of all test material in open dishes and in nylon bags provided that it had been directly moistened with water before sterilization Even test material wrapped in nylon foil together with a moist swab was sterilized in practically all cases This converted autoclave is particularly suitable when large numbers of articles or bulky objects are to be sterilized

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IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS-REACTIONS IN THE KLEBSIELLA GROUP

9. Cross Reactions between *Klebsiella Types 11, 21 and a Closely Related Strain*

By

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Received 22.xii.62

In a previous study (1) a klebsiella strain, W60, *originally isolated* from water, was found to give capsular reactions up to the homologous titre in immune sera against the sero types 11 and 21. The reactions obtained were the following:

Strain	Immune serum anti 11	anti 21
Type 11	16	16
Type 21	4	32
W60	32	32

After absorption of these two immune sera with the *strain* W60 the homologous antigens no longer showed a detectable capsular *reaction*. The results, therefore, suggested the possibility that the strain W60 possessed the type-specific antigens of both types and thus *might be a "double"* type similar to the artificial double types *of Haemophilus* influenzae, produced by *Leidy, Hahn & Alexander* (2), and of *Diplo*coccus pneumonia, produced by *Austrian et al.* (3–6). This paper reports the results of a quantitative study of these cross-reactions.

MATERIAL AND METHODS

The type strains of types 11 and 21 in the following designated strains 11 and 21 were originally received from *Dr. F. Kauffmann*, Copenhagen. Strain W60 was isolated from a sample of drinking water. All strains had been kept in the lyophile state for some years.

Preparation of immune sera and serological methods were as described previously (7).

Capsular polysaccharides were prepared from bacterial suspensions in *water by* a simplified method described *in* a previous paper (8). Qualitative chemical analysis was carried out by paper chromatography as described previously.

Agar diffusion precipitin analysis was carried out as described by *Ouchterlony* (10). Immune serum was put in a central well and antigens were *distributed* in 6 peripherical wells. In some instances the wells were refilled when empty.

RESULTS

The composition of the capsular polysaccharides is shown in Table 1 The polysaccharides of strains 11 and W60 have the same composition whereas strain 21 lacks glucose, which is present in the two others

The results of capsular reactions are shown in Table 2 The results confirm that the capsular polysaccharides of the three strains are closely similar serologically, but in these sera there are some differences between strain 21 and the two others

The results of quantitative precipitin determinations are shown in Figs 1 to 3 The nitrogen contents of the antigens used in these tests were 0 3 per cent (11, W60) and 0 2 per cent (21) The antigens 11 and W60 precipitate equal quantities of antibody from serum anti 11, whereas strain 21 precipitates only 42 per cent of the total antibody From serum anti-21 the antigens 11 and W60 bring down about one half of the total precipitable antibody, W60 slightly more (57 per cent) than 11 (47 per cent) The difference is small, but suggestive when seen together with the results, obtained with the serum anti-W60 In this serum antigen 11 reacts only with 80 per cent of the antibody, and antigen 21 precipitates 32 per cent

The results obtained with the gel diffusion method are illustrated in Figs 4 to 6 Fig 4 indicates that the serum 11 contains at least two different antibodies, one of which reacts with an antigen shared by strains 11 and W60, but apparently not present in antigen 21 The reactions of antigens 11 and W60 have the appearance of reactions of

TABLE 1

Chemical Composition of Capsular Polysaccharides Isolated from Klebsiella Strains 11 21 and W60

Strains	11	21	W60
Uronic acid	+	+	+
Glucose	+	0	+
Galactose	+	+	+
Mannose	+	+	+

TABLE 2

Capsular Reactions of Klebsiella Strains 11 21 and W60 in Immune Sera against the same Strains

Antigen	Immune serum		
	Anti 11	Anti 21	Anti W60
11	128*	16	64*
21	32	64*	32
W60	128	16	64*

Titres are given as the reciprocal of the highest serum dilu giving positive reaction
* Only weak reactions with the highest serum dilution

identity, and with this arrangement of the wells there is no indication of any dissimilarity between these two antigens The second antibody, which produces a much less distinct precipitate band, reacts with an antigens which appears to be present in all three strains, since an apparently complete ring of precipitate is formed around the central well

The results obtained with serum anti-W60 (Fig 5) are less easy to explain, but suggest the presence of at least 2 antibody fractions One of these reacts with an antigen common to strains 11 and W60, and produces a precipitate band which may correspond to the peripherical band in Fig 4 Like the latter, it appears to show a reaction of identity The second band runs parallel to the first band except where it ap-

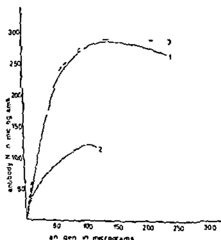


Fig 1

Quantitative precipitin determinations in serum anti 11 Curve 1 antigen 11 Curve 2 antigen 21 Curve 3 antigen W60

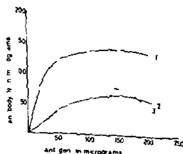


Fig 2

Quantitative precipitin determinations in serum anti 21 Curve 1 antigen 21 Curve 2 antigen 11 Curve 3 antigen W60

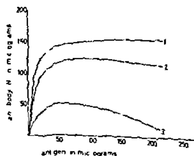


Fig 3

Quantitative precipitin determinations in serum anti W60 Curve 1 antigen W60 Curve 2 antigen 11 Curve 3 antigen 21

Fig 4
Gel precipitation test with serum anti 11 (central well) and the three antigens (peripheral wells)

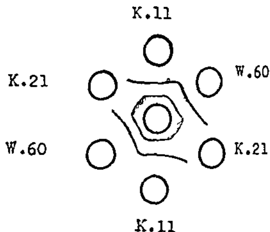


Fig 5
Gel precipitation test with serum anti W60 (central well) and the three antigens (peripheral wells)

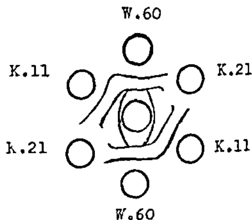
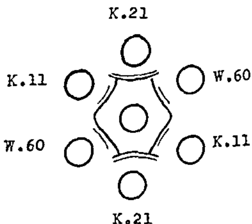


Fig 6
Gel precipitation test with serum anti 21 (central well) and the three antigens (peripheral wells)



proaches to the well containing antigen 21 Here the ends of the bands bend towards one another At first sight, this might seem to suggest the presence of small quantities of the same antigen in strain 21, but since no visible precipitate is seen in the vicinity of the wells containing antigen 21, the effect is probably due to the advancing boundaries of antigen fractions from strains 11 and W60 only More interiorly, towards the central well precipitate bands are seen opposite to the wells containing antigen 21 These bands fuse with a part of the bands just described, indicating a reaction of partial identity between an antigen contained in strain 21 and an antigen common to the two other strains Thus the reaction of antigen 21 in this serum seems to be due to partial cross reactivity of a fraction of this antigen with one of the antigen fractions of the homologous strains

Fig 6 finally shows that antigen 21 contains two fractions, one of which shows a reaction of partial identity with an antigen common to strains 11 and W60

There is a suggestion of a slight reaction between a second antigen fraction contained in strains 11 and W60 and this serum, but these precipitate bands were very faint, and the reactions may not be reliable

DISCUSSION

The results confirm that the three strains show marked cross reactions in the immune sera against one another, but do not confirm that the strain W60 was a double type, as suggested by previous results Two reasons for this discrepancy might be suggested One is that capsular reactions carried out after absorption of the sera with cross-reacting bacterial antigens may be difficult to read due to decreased transparency of the absorbed sera Thus remaining reactivity of the serum used in previous experiments may have been overlooked The second is that the cross reactions obtained with immune sera from different rabbits may differ

The two strains 11 and W60 appear to produce nearly identical capsular antigens Indeed the results of gel precipitation tests suggest that th
ive precipitin deter-
the two strains It
ie to an additional
antigen in the preparation produced from strain W60, an antigen present in too small quantity to be detectable by gel precipitation If so, these two strains may be considered to belong to the same capsular type It also seems possible that the difference might be due to minor structural differences between the reactive groupings on one
other pair of
case a reac
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The resu ... that the antigen preparation from each of the three

strains contains at least two distinct antigens This is in agreement with results obtained in a study of the capsular antigen of Klebsiella sero-type 1 (11) It is not yet known whether this signifies that the capsule of such strains is composed of two polysaccharides, or that some other antigen, *e g* a somatic antigen has contaminated the capsular antigen In order to decide this it will be necessary to separate the two antigens and to study their rôle in the capsular reactions of the strains

These results indicate that the antigenic constitution of Klebsiella strains may be more complicated than expected

SUMMARY

Studies on the capsular antigens of three cross-reacting Klebsiella strains are reported Two separate antigens could be detected in the antigen preparations from each of the three strains

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NON-PIGMENTED SERRATIA MARCESCENS VAR. KIELENSIS AS A PROBABLE CAUSE OF BRONCHOPNEUMONIA

By

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The normal habitat of Serratia is water and soil. While considered to be a strict saprophyte in the past, there is now growing evidence for its capability of producing human infection, especially in chronically debilitated patients (9, 10). Serratia has been found to be associated with skin and wound infections, infant diarrhea, infections of the urinary and respiratory tracts, sinusitis, otitis media, meningitis, endocarditis and septicaemia (7, 16, 20, 21 and other references cited). In some of the reported cases the pathogenetic rôle of Serratia may be questioned, but it seems wise to recognize this group of organisms as potential cause of serious infection, particularly since the advent of the widespread use of antibiotics capable of altering the bacterial flora of the human body, setting the stage for enhanced invasiveness and virulence on the part of these bacteria (2).

Next to the urinary tract the most frequently involved organ system in Serratia infections seems to be the respiratory tract.

We have found reports on only two cases of pneumonia considered to have been due to Serratia. *Aitoff et al.* 1936 (1) reported pure culture of ratia from sputum in a case of pneumonia. *Bernhard & Sutton* 1962 (2) described a patient who became ill with Serratia pneumonia after 10 days of treatment with penicillin and tetracycline of a pelvic abscess caused by Staphylococcus pyogenes var. aureus.

Isolation of Serratia in chronic bronchitis has been described by *Woodward & Clarke* 1913 (22), *Gale & Lord* 1957 (8) and *Robinson & Woolley* 1957 (18). *Gale & Sonnenwirth* 1962 (9) reported mild respiratory infection possibly caused by Serratia in a case with previous lobectomy for bronchiectasis. Mild respiratory disease in laboratory workers exposed to aerosols of Serratia marcescens, has been reported by *Paine* 1946 (15) and by *Reitman et al.* 1955 (17).

The present publication is justified by the scarceness of previous reports on Serratia infections. In addition, the isolated Serratia variety is of interest because of its very rare identification in human materials

and because there was need for an extended examination to clarify the bacteriological diagnosis

CASE REPORT

T O 64 years old The patient had a severe influenza in 1918 Since then frequent attacks of upper respiratory disease have occurred involving cough expectoration and slight fever In 1955 he had an attack of thrombosis of the right carotid artery with left hemiparesis sequelae of which still persist His cough reflexes are weak

In the summer of 1961 he had bronchophneumonia and recovered after 14 days in bed In the middle of December 1961 he became ill again with fever, cough and yellow expectoration He was admitted to hospital on December 27, after having been treated with penicillin On admission his general condition was poor Tp 38 7 Leucocyte count 26100 SR 90 mm Total serum proteins 5 4 g per 100 ml with reduction of albumin and elevation of α_1 and α_2 globulins Clinical and radiological examinations revealed leftsided lung infiltration and pleural effusion Diagnosis Bronchopneumonia

Penicillin and streptomycin treatment during the first days in hospital had little effect On January 1 1962 chloromycetin treatment was started The temperature then gradually returned to normal together with general and radiological improvement The leucocyte count decreased to 11300 on January 9 SR rose to 98 mm on January 1 14 days later it was 80 mm When the patient left hospital on January 20 1962 he was in a good condition One year later he is still healthy

MATERIAL

Bacteriological specimens Expectorations sampled on December 28 December 31 and January 5 were subjected to common bacteriological examination (Specimens sampled on December 31, January 1 and January 2 were cultured for tubercle bacteria with negative results)

Blood specimens Agglutination tests with patient sera were performed with blood sampled on January 5 15 and 20 1962

METHODS

Primary cultures were made aerobically on blood agar plates and subcultures on
' tes
r the formation of acid from carbohydrates and
in 1 per cent peptone water to which had been
added 1 per ... by means
of paraffin ... r This
procedure al ... bolism
The absence of gas production in glucose and mannitol was controlled by means of Durham tubes

Reaction in d tartrate tests for nitrate reduction and indol production and methyl red and Voges Proskauer reactions were carried out according to *Kauffmann* (12) Utilization of citrate as the sole source of carbon was read in the conventional Koser s medium gelatin liquefaction in a medium of 15 per cent gelatin in broth and hydrogen sulfide production in TSI medium (Difco) The urease test was performed according to *Blake Christensen* (3) the KCN test according to *Moller* (14) glutamic acid decarboxylase activity test according to *Shaw & Clarke* (19) by their simplified technique and the phenylalanine reaction according to *Henriksen & Closs* (11) by a slightly modified procedure (19)

Oxidase reaction was performed with a 1 per cent aqueous solution of dimethyl p phenylenediamine hydrochloride and with a 0 5 per cent freshly prepared aqueous solution of tetramethyl p phenylenediamine hydrochloride the catalase reaction with a 3 per cent hydrogen peroxide solution and ether sensitivity test in the following manner 1 volume ... as shaken with 2 volumes of ether for 1 min ... d and blood agar heavily inoculated from th ... read on Loeffler serum slants and litmus ... r cent litmus in skimmed milk

Flagellar staining was performed as described by *Leifson* with formaldehyde fixation (4), and antibiotic sensitivity tests according to the method of *Ericsson et al* (6)

Serological procedure An experimental antiserum was obtained by intravenous injection into a rabbit of 0 25 0 5 1 and 2 ml of a moderately turbid suspension of washed living bacteria from 6 hr old cultures on nutrient agar surfaces Injections were undertaken at 5–7 days' intervals and bleeding 7 days after the last injection This serum was compared with the 3 patient serum specimens in agglutination tests

RESULTS

Cultural characteristics All three primary cultures were pure and abundant Single colonies on blood agar were roughly circular, measuring up to 3 mm in diameter after 24 hr at 37° C They were convex and not swarming As colonies aged, they showed a great tendency to form papillae They were densely opaque and their consistency was butyrous There was a slight smell of ammonia Single colonies were non haemolytic, but heavier growth zones revealed pronounced haemodigestion Growth on blood agar became brownish green when aged On no occasion red pigment production was observed On bromothymol blue lactose agar colonies were bluish green After several days on this medium at room temperature a yellowish tinge of colonies was observed, while the medium was rendered more and more alkaline Sectors or "tongues" of these old yellowish colonies were light blue in colour In subculture these mutants showed no haemodigestion and were less active than the [illegible]

[illegible] reactions, except for lactose fermentation, which in the mutants could not be detected aerobically in 30 days (KCN test and glutamic acid decarboxylase test were not performed with the mutants)

Growth in liquid media was diffuse, without pellicle and with little sediment in young cultures

Growth optimum was approximately 32–34° C, but abundant growth occurred at all temperatures between 28 and 37° C Growth at 22° was willing, but less abundant There was no growth at 41° C, and no survival after heating to 56° C for 30 minutes The strain was a facultative anaerobe, but growth anaerobically was strongly depressed

Microscopical morphology The strain was invariably Gram negative, not acid fast, and not sporulating Some variation in [illegible]

[illegible] coccobacilli intermingled by thin rods Indian ink preparations made a narrow capsule evident Motility in most [illegible]

[illegible] shape, which has been described by *Leifson* in Serratia (13)

TABLE 1

Biochemical Behaviour

Adonitol	+ (3–4)	Raffinose	— (30)
Dulcitol	— (30)	D tartrate	— (14)
Sorbitol	+ (1)	KNO_3 reduction	+ (1)
Arabinose	— (30)	V P 1 day	—
Xylose	— (30)	V P 4 days	—
Rhamnose	— (30)	M R 1 day	+
Maltose	+ (1)	M R 4 days	+
Salicin	+ (1)	V P 2 days 22° C	—
Inositol	+ (1)	M R 2 days 22° C	+
Lactose	+ (10 20)	Indol production	— (4)
Sucrose	+ (1)	H_2S production	— (4)
Mannitol	+ (1)	Gelatin liquef	+ (1)
Glucose	+ (1)	Serum liquef	+ (1)
Trehalose	+ (1)	*Citrate growth*	+ (1)
Mannose	+ (1)	Urease production	— (30)
Fructose	+ (1)	Phenylalanine reaction	—
Galactose	+ (1)	KCN test	+
Dextrin	+ (1)	Glutamic acid decarb	+
Starch	+ (1)	Ether sensitivity	+
Glycogen	— (30)	Catalase production	+
Inulin	— (30)	Oxidase reaction	—
Glycerol	+ (1)	Litmus milk Acid with clot in 1 day	
Cellobiose	— (30)	Digestion of clot in 5 7 days	

In fermentation reactions + or — indicate acid or no detectable acid production aerobically Added figures in brackets indicate time in days for reactions to appear or days of incubation in negative reactions Incubation temperature 37° C if no other temperature is mentioned

TABLE 2

Tube Agglutination with three Patient Sera and one Rabbit Antiserum

Antigen	Sera A	B	C	D
5 hr broth culture living bacteria	320	320	320	10240
5 hr broth culture heated to 60° C for 1 hr	*0*	*0*	*0*	*640*
20 hr agar culture suspension heated to 100° C for 2 hr	0	0	0	320
20 hr agar culture suspension heated to 120° C for 2 hr	0	0	0	320

Doubling dilution from 1 in 10 Titres listed are inverse of last distinctly positive dilution A C are patient sera sampled on January 5 15 and *20 1962* respectively D is rabbit antiserum to living bacteria

Biochemical reaction See Table 1 The strain was invariably anaerogenic in fermentation reactions also when tested at *22° C* When aerobic tubes turned acid there was also pronounced acid reaction anaerobically which made a fermentative mode of metabolism evident

The strain was forwarded to Dr *P R Edwards* Communicable Disease Center Atlanta Georgia He reports that Dr *W H Ewing* considers *it to be a typical strain of* Serratia marcescens var kiliensis

Serology Patient sera gave a moderate and somewhat slow agglutination in preliminary slide agglutination tests In Table 2 results of tube agglutination with patient and rabbit antisera are listed

Pathogenicity to laboratory animals White mice injected intraperitoneally with 10^8 living bacteria washed in saline died in 1 to 3 days On the other hand the rabbit immunized with living bacteria intravenously did not show any sign of illness during one month

Chemotherapeutic sensitivity The strain was highly sensitive to sulphonamide streptomycin and chloromycetin moderately sensitive to tetracyclines and resistant to penicillin kanamycin erythromycin and polymyxin B

DISCUSSION AND CONCLUSION

The identification of the Voges Proskauer negative Serratia marcescens var kielensis is extremely rare in medical bacteriology (5) In the scarce literature on Serratia as possible human pathogen this variety has not been encountered However as the lack of readily visualized pigment production is very frequent in Serratia (5 7) it is probable that many cultures of Serratia have in the past been classified as Aerobacter or intermediate coliform bacteria (5) An exact estimate of the distribution and general importance in human pathology of Serratia including the kielensis variety is therefore impossible at present

That our strain was found in abundant pure culture in three successive specimens from a case of clinical infection which responded well to the antibiotic treatment indicated in vitro are strong arguments for its pathogenicity and causal importance Supporting evidence for this is also the observed antibody response The stability of the antibody titre during 15 days may be explained by the probably longstanding illness The titre was rather low but this may in part be explained by the generally poor condition of the patient As a matter of fact lowered resistance may have been decisive for the infection

The antigen reacting with patient sera did not withstand 60° C for 1 hr resembling the L antigen of Escherichia and the Vi antigen of Salmonella with regard to thermolability A rabbit antiserum to living bacteria showed a similar peak of antibodies to a thermolabile antigen which showed the agglutinogenic capacity of the latter The observed thermolability of this probably flagellar antigen may have connection with heat influence on the flagellar morphology

SUMMARY

lat ... as iso
ch ... bron
... otic treatment of choice according to in vitro tests There was a specific antibody response in patient sera It is concluded that the strain probably had an actual pathogenetic importance in the case reported

TABLE 1

Biochemical Behaviour

Adonitol	+ (3 4)	Raffinose	— (30)
Dulcitol	— (30)	D tartrate	— (14)
Sorbitol	+ (1)	KNO_3 reduction	+ (1)
Arabinose	— (30)	V P 1 day	—
Xylose	— (30)	V P 4 days	—
Rhamnose	— (30)	M R 1 day	+
Maltose	+ (1)	M R 4 days	+
Salicin	+ (1)	V P 2 days 22° C	—
Inositol	+ (1)	M R 2 days 22° C	+
Lactose	+ (16 20)	Indol production	— (4)
Sucrose	+ (1)	H_2S production	— (4)
Mannitol	+ (1)	Gelatin liquef	+ (1)
Glucose	+ (1)	Serum liquef	+ (1)
Trehalose	+ (1)	Citrate growth	+ (1)
Mannose	+ (1)	Urease production	— (30)
Fructose	+ (1)	Phenylalanine reaction	—
Galactose	+ (1)	KCN test	+
Dextrin	+ (1)	Glutamic acid decarb	+
Starch	+ (1)	Ether sensitivity	+
Glycogen	— (30)	Catalase production	+
Inulin	— (30)	Oxidase reaction	—
Glycerol	+ (1)	Litmus milk Acid with clot in 1 day	
Cellobiose	— (30)	Digestion of clot in 5 7 days	

In fermentation reactions + or — indicate acid or no detectable acid production aerobically Added figures in brackets indicate time in days for reactions to appear or days of incubation in negative reactions Incubation temperature 37° C if no other temperature is mentioned

TABLE 2

Tube Agglutination with three Patient Sera and one Rabbit Antiserum

Antigen	Serum			
	A	B	C	D
5 hr broth culture living bacteria	320	320	320	10240
5 hr broth culture heated to 60° C for 1 hr	0	0	0	640
20 hr agar culture suspension heated to 100° C for 2 hr	0	0	0	320
20 hr agar culture suspension heated to 120° C for 2 hr	0	0	0	320

Doubling dilutions from 1 in 10 Titres listed are inverse of last distinctly positive dilution A C are patient sera sampled on January 5 15 and 20 1962 respectively D is rabbit antiserum to living bacteria

Biochemical reaction See Table 1 The strain was invariably anaerogenic in fermentation reactions, also when tested at 22° C When aerobic tubes turned acid, there was also pronounced acid reaction anaerobically, which made a fermentative mode of metabolism evident

The strain was forwarded to *Dr P R Edwards Communicable Disease Center Atlanta Georgia He reports that Dr W H Ewing* considers it to be a typical strain of Serratia marcescens var kielensis

Serology Patient sera gave a moderate and somewhat slow agglutination in preliminary slide agglutination tests In Table 2 results of tube agglutination with patient and rabbit antisera are listed

Pathogenicity to laboratory animals White mice injected intraperitoneally with 10^9 living bacteria washed in saline died in 1 to 3 days On the other hand the rabbit immunized with living bacteria intravenously did not show any sign of illness during one month

Chemotherapeutic sensitivity The strain was highly sensitive to sulphonamide streptomycin and chloromycetin moderately sensitive to tetracyclines and resistant to penicillin kanamycin erythromycin and polymyxin B

DISCUSSION AND CONCLUSION

The identification of the Voges Proskauer negative Serratia marcescens var kielensis is extremely rare in medical bacteriology (5) In the scarce literature on Serratia as possible human pathogen this variety has not been encountered However as the lack of readily visualized pigment production is very frequent in Serratia (5 7), it is probable that many cultures of Serratia have in the past been classified as Aerobacter or intermediate coliform bacteria (5) An exact estimate of the distribution and general importance in human pathology of Serratia including the kielensis variety is therefore impossible at present

That our strain was found in abundant pure culture in three successive specimens from a case of clinical infection which responded well to the antibiotic treatment indicated in vitro are strong arguments for its pathogenicity and causal importance Supporting evidence for this is also the observed antibody response The stability of the antibody titre during 15 days may be explained by the probably longstanding illness The titre was rather low but this may in part be explained by the generally poor condition of the patient As a matter of fact lowered resistance may have been decisive for the infection

The antigen reacting with patient sera did not withstand 60° C for 1 hr resembling the L antigen of Escherichia and the Vi antigen of Salmonella with regard to thermolability A rabbit antiserum to living bacteria showed a similar peak of antibodies to a thermolabile antigen which showed the agglutinogenic capacity of the latter The observed thermolability of this probably flagellar antigen may have connection with heat influence on the flagellar morphology

SUMMARY

A non pigmented strain of Serratia marcescens var kielensis was isolated repeatedly in pure culture from expectorations in a case of bronchopneumonia which responded well to the antibiotic treatment of choice according to in vitro tests There was a specific antibody response in patient sera It is concluded that the strain probably had an actual pathogenetic importance in the case reported

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IMMUNOELECTROPHORETIC ANALYSIS OF BLOOD STAINS WITH SPECIAL REFERENCE TO Gc GROUPING

By

B NERSTROM and J SKAFTE JENSEN

Received 11.xii 62

Grouping of blood stains, which is of great importance within forensic medicine, has in practice until recently been possible with regard to the ABO system only

Adoption of new principles such as the mixed agglutination (1) and adsorption elution principle (5) has not only enabled ABO grouping of very small stains, but also allowed demonstration of the M , N-, and D-factors in stains (7) However, elaboration as to the number of applicable systems would be of significant value

Reports on the demonstration of haptoglobin groups in blood stains (2) as well as preliminary results of Gm grouping of blood stains in this laboratory (3) seem to indicate that the genetically determined polymorphism of the serum proteins may become an important part of the analysis of blood stains

This work presents the results of some preliminary immunoelectrophoretic examinations of blood stains with a view to Gc grouping

MATERIAL AND METHODS

Blood Stains on Fabrics

Examinations were made of a number of stains on linen stained with whole blood of 3 donors of types Gc 1–1, Gc 2 1 and Gc 2–2 respectively The stains originating from the Gc 1–1 donor were examined after 2 days of storage, the stains from the Gc 2–1 donor after 3 days and the stains from the Gc 2–2 donor after 2, 6 and 7 days From the Gc 1–1 donor further stains, consisting partly of whole blood, partly citrated blood were made on linen and silk and examined when 3 hours old

All stains were allowed to dry and were stored at room temperature

Blood Stains on Filter Paper

Of 4 sets of filter paper strips 2 × 2 mm (Whatman no 3) one was moistened with 3 microliters of blood from the above donor of type

Gc 1-1, another from the donor of type Gc 2-2 and the remaining two with blood from 2 new donors of type Gc 2-1 The stains were left to dry and stored at room temperature With a view to a more systematic study of the storage period's influence on Gc-grouping, the examination of stains from the 4 sets was performed at increasing intervals, from after 4 hours and up to 15 days of storage

Serum Stains on Filter Paper

Finally, sets of filter paper strips 2 × 2 mm were moistened with 1½ microliter serum from 2 donors of type Gc 2-1 and 2-2 The strips were stored at room temperature and examined at increasing intervals after storage from 1 to 55 days

Immunoelectrophoresis

The employed technique is described in detail in an earlier publication (6) The adaptation of the technique to examination of the serum proteins in blood- and serum stains required few modifications only

The blood- and serum stains on filter paper were examined by insertion of the filter paper strips into the agar at the site of the antigen basin

In some cases blood stains on fabric were examined in the same way by insertion of a 2 × 2 mm piece of the fabric into the agar In other cases a stain eluate was made and applied to the antigen basin in a quantity of 1 to 2 microliters

For the elution saline 0 9 per cent as well as distilled water were used and the process was carried out at room temperature over a period of from 1 to 24 hours In the first examinations the quantity of the solvent was calculated on the basis of the dry matter weight within the particular stain under examination in an attempt to maintain the same dry matter content in the eluate as in blood (about 10 per cent) However in later experiments stains of 1 2 cm² were soaked with as small a quantity of solvent as possible and the eluate was squeezed out of the material

In a few cases a further concentration was attained by evaporation of the eluate onto a 2 × 2 mm filter paper strip used for the insertion into the agar

At the immunoelectrophoretic examination the stain material was placed opposite a serum reference of a known Gc type

The distance between the antigen basin and the antiserum trough was usually 5 mm but experiments have been carried out with distances as short as 1 mm

The presence of small amounts of Gc substance in a stain eluate was demonstrated by *an absorption test performed on an agar slide* furnished with a centrally placed antigen basin containing reference serum and two lateral troughs one holding immune serum the other equal parts of immune serum and stain eluate

Three different antisera were used (R 146, R 214 and R 225) Each antiserum was produced at the laboratory by injection of human serum into rabbits

The results were read from the native as well as from the amido-black stained preparations Each native preparation was photographed according to the socalled indirect method

RESULTS

Blood Stains on Fabrics

The analysis of blood stains on different fabrics displayed immunoelectrophoretic patterns, which for the majority of the precipitates did not deviate from normal serum patterns In fresh stains the symmetry with the serum reference was complete, whereas in older stains a certain decrease was noted in the number of arcs as well as a cathodic extension of some arcs, most pronounced in the case of the albumin arc

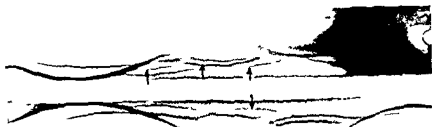


Fig 1

Immunoelectrophoretic pattern of a blood stain on silk stored for 3 hours and originating from a donor of type Gc 1 1 (above) Serum from the same donor (below) Arrows indicate the Gc the new alpha 1 globulin and the reaction of immunological identity Antiserum B 146

These trends, however, did not hold true for the Gc precipitates In 3 hour old stains from a donor of type Gc 1–1 a distinct Gc 1–1 precipitate was found, but the distance between the precipitate and the immune serum trough was increased and the precipitate showed further characteristics of reduced concentration Co-existing, in the alpha-1 globulin region, a precipitate occurred with which the Gc precipitate formed an identity reaction (Fig 1) In 2 day old stains from the same donor a weak Gc 1–1 precipitate was demonstrable using stain eluate for the application whereas no normal Gc precipitate was displayed by direct insertion of the stain material into the gel In both cases the new arc occurred in the alpha 1 globulin region

In 2 day-old stains from a donor of type Gc 2–2 a questionable Gc 2–2 precipitate was found at a considerable distance from the immune serum trough and a new arc similar to the one described above appeared in the alpha-1 globulin region

In 3-day-old stains (type Gc 2–1) as well as in 6 and-7 day-old stains (type Gc 2 2) no Gc precipitate could be demonstrated, but the new arc in the alpha-1 globulin region was observed Through absorption with an eluate from the 3 day-old stain of type Gc 2–1 anti Gc was removed from the immune serum

Fig 2

Immunoelectrophoretic pattern of blood stains on filter paper stored for 13 hours and originating from donors of type Gc 1 1 (A) and Gc 2 2 (C) opposite corresponding sera (B and D) Antiserum R 146

Blood Stains on Filter Paper

Examination of blood stains on filter paper was performed at brief intervals beginning with hours' and increasing to days' intervals between tests The Gc precipitates were found to be weakened in stains

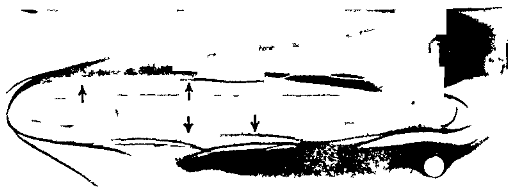


Fig 3

Immunoelectrophoretic pattern of a blood stain on filter paper stored for 48 hours and originating from a donor of type *Gc 2 1 (above) Serum from the same donor* (below) Antiserum R 214

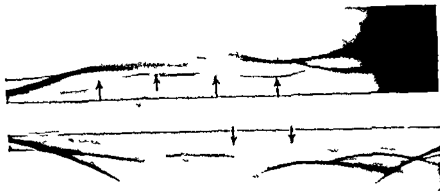


Fig 4

Immunoelectrophoretic pattern illustrating the establishment of the immunological identity reaction between a weak Gc 2 component and the "new alpha 1 globulin" by admixture of serum of type Gc 1-1 to a blood stain on filter paper stored 48 hours and originating from a donor of type Gc 2-2 (above). Serum reference of type Gc 2-1 (below). Antiserum R 146

less than 24 hours old. Reliable Gc grouping was only possible within the first approximately 2 days and then only of Gc 1-1 and Gc 2-2 stains (Fig 2), the Gc 2-1 precipitates appearing non-characteristic, asymmetric with a severely weakened 2 component (Fig 3).

In a few older stains of type Gc 1-1 the Gc type was recognizable.

In nearly all cases co-existing with the weakening of the Gc precipitates the new arc observed by the examination of the stains on fabrics occurred in the alpha 1 globulin region. The immunological reaction between the Gc precipitate and the new arc was most distinct in the stains of type Gc 1-1 and 2-1. In the stains of type Gc 2-2 the

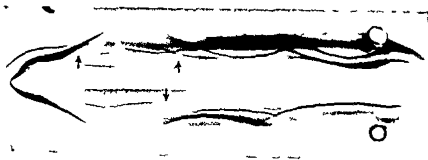


Fig 5

Immun…
Above…
glob…
by qu…

identity reaction was sometimes weak, but could be established by adding serum of type Gc 1–1 to the stain (Fig 4)

Serum Stains on Filter Paper

In serum stains the Gc substance was much more stable Flattening and blurring of the Gc precipitates did not occur until after about 1 month's storage, *i e* in time with the other arcs in the preparation The deterioration was particular pronounced in the case of the Gc 2–1 precipitates The experiment was extended to 2 month old stains, in which weak and non-characteristic Gc precipitates appeared

The above mentioned precipitate in the alpha-1 globulin region, occurring in the blood stains, was not observed in serum stains on filter paper

DISCUSSION

A total of more than 100 individual examinations of blood stains on fabric were performed, but owing to the variety of fabrics, methods of application etc the number of uniform stains examined with the same technique is too small for a detailed analysis of the results

However,it can be established that the Gc 1 1 and 2–2 types were demonstrable in blood stains a few days old Further that a new precipitate in the alpha-1 globulin region showing immunological identity to Gc appears as the normal Gc precipitates are weakened and disappear The ability of the stain eluate to absorb anti Gc was shown to be preserved in spite of the disappearance of the normal Gc precipitates Investigations of blood stains on filter paper have yielded results largely corresponding with these findings

Gc 1 1 precipitates have been demonstrable after storage of longer duration than Gc 2–2 precipitates, while Gc 2–1 precipitates have been found asymmetric showing a relative weak 2-component Whether these findings are due to coincidence, are a characteristic feature of the samples employed, or are due to a weaker resistance on the part of the 2 component towards the effect of storage, can only be decided through more extensive examinations

In contradistinction to the results of the examination of blood stains it has been possible to determine the Gc group of serum stains of approximately the same serum protein content as the blood stains after storage for several weeks The present investigation does not offer any explanation of this difference between blood- and serum stains

With regard to the new alpha-1 globulin component immunologically identical with Gc it should be emphasized that its electrophoretical position was independent of the Gc type of the stain Further *it may be* mentioned that the precipitate was observed with the use of different antisera having in common a powerful anti Gc The precipitate was only demonstrated in blood stains *not in serum stains In this connec-*

tion, however, it should be mentioneed that a similar precipitate has been observed in a few sera among a number of poor quality samples from Greenland even though the serum has been separated from the blood cells in Greenland An example is given in figure 5 The immunoelectrophoretic pattern of so called pathologic serum as shown by *Hirschfeld* (5) manifests a precipitate with a similar position

It appears that the Gc substance during unfavourable storage and under the influence of unknown factors undergoes a gradual transformation into a substance with an electrophoretical mobility like that of an alpha 1 globulin without losing its immunological specificity It is found that this transformation takes place rapidly in dried blood stains, but nothing is known about the nature of this so called transformation A partial decomposition or binding to decomposition products in the serum can be mentioned as possibilities

SUMMARY

Immunoelectrophoretic examination has been performed of a limited number of blood stains on different fabrics as well as of blood-and serum stains on filter paper The Gc groups of serum stains were demonstrable after storage at room temperature for 1 to 2 months The Gc substances in blood stains were found to be very labile, the Gc precipitates presenting a rapid weakening and disfiguration The appearance of a precipitate in the alpha 1 globulin region immunologically identical with the Gc precipitates was demonstrated The results are discussed and it is concluded that the Gc under unfavourable storage and particularly in blood stains may undergo transformation into a faster migrating but immunologically identical component

The extent of the present study does not allow any evaluation of the [illegible]

[illegible] test is only applicable to completely fresh stains

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4 [illegible]

5 [illegible]

6 [illegible] the inheritance of the Gc [illegible]

7 [illegible]

FROM THE UNIVERSITY INSTITUTE OF FORENSIC MEDICINE (HEAD PROFESSOR HARALD GORMSEN, M D), DEPARTMENT OF SEROLOGY (HEAD K HENNINGSEN, M D), COPENHAGEN, AND THE MATERNITY WARD, DEPARTMENT B RIGSHOSPITALET, COPENHAGEN (HEAD PROF EBBE BRANDSTRUP M D)

ON THE PRESENCE OF GROUP-SPECIFIC Gm- AND Gc-SUBSTANCES IN URINE

By

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Received 11 xii 62

Serum grouping according to the genetically determined Gc- and Gm systems is based upon serologically and immuno-electrophoretically demonstrable polymorphism within the alfa-2- and gamma globulin fractions of the serum proteins

As certain biological fluids contain serum proteins, though in varying quantities, serum grouping according to the systems in question might be possible As regards the Gc-system the group-specific substance has been demonstrated in certain samples of cerebrospinal fluid, in ascitic fluid and extravasates (9) Concerning the Gm-system similar reports are not available, but in a few samples of synovia from knee joints one of us (J C N) has been able to demonstrate the Gm type of the patients

As even normal urine contains traces of serum proteins (11) including the alfa-2 and gamma globulin fractions (16, 2, 15), it might be possible to demonstrate Gc and Gm substances in urine *Hirschfeld's* (9) suggestion of the presence of Gc substance in urine agrees with this assumption

The present paper is based upon serological and immuno-electrophoretic examination of a small number of concentrated normal and proteinuric urine samples with a view to the occurrence of group-specific Gm and Gc substances in urine

MATERIALS AND METHODS

Urine 1) Samples of normal human urine were supplied by healthy adults (members of the laboratory staff) 2) Samples of proteinuric urine were furnished by pregnant women from the Maternity Ward Department B Rigshospitalet Copenhagen during the experimental period The only criterion applied to the selection of the patients was the presence of proteinuria demonstrable by *Heller's test* The degree of proteinuria as estimated by the Esbach test varied from 0 3 10 per thousand The urine which was passed under simple precautions to avoid contamination was collected during a period of 12 24 hours The cause of proteinuria in all cases was preeclampsia from mild to severe degrees none of the cases

presenting a basis for the assumpti n that any chronic diseases of the heart or the kidneys were manifest The age of the patients ranged from 17 to 37 years Six of the patients were I parae two II parae one IV para and one V para

Process of concentration The urine was concentrated by dialysis The volumes of urine employed for concentration in the first experiments were 40–50 ml In later

merthiolate was added both to saline and dextran solutions The degree of concen

not taking into c nsiderati n the non prote n nitrogen[1] Determination of the fractions of serum prote n were based upon paper electrophoresis Total serum prote n was determined by the Biuret test

Gm Group Determination

The principle of the group determination is a *specific inhibition of different* aggl t nat ng

Gm Technique Of the urine concentrate series of doubling dilutions 1 1 1 32 were ma le The

microscopy Agglutination was graded as + ++ +++ ++++ Complete inhib tion of the agglutination was designated —

Gc Group Determination

The C

c electrophoretic duration 2 hours voltage 7V/cm) an immune serum was added to the trough between the two basins *upon which* diffusion took place for 24 hours at 37° C

The immune serum concerned a rabbit anti h iman

pe

in

of

tr concen

Verif cation test as to the *identity of the Gc* precipitates of the urine was performed according to the principles described by *Hirschfeld* (10) *Production* of identity reaction between *Gc of ur ne* proteins and Gc of serum proteins was attempted by variation in the *length* of the immune serum trough as well as by production of a horse shoe shaped trough around 2 antigen basins *one charged* with urine concentrate the *other* with serum

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[1] Thanks are due to *Poul Astrup* M D Rigshospitalet Copenhagen who kindly performed the Kjeldahl nitrogen analyses

RESULTS

The samples of normal urine were concentrated approximately 100 times as estimated by control weighings, but protein concentrations detectable by Kjeldahl's nitrogen analysis were not obtained The samples of proteinuric urine were concentrated 5–50 times This marked variation was due to differences in the duration of the dialysis against dextran as well as differences in the protein contents of the samples prior to concentration The final protein contents of the proteinuric urine samples ranged from 1 1–12 1 per cent This variation in the protein contents made it possible to estimate the sensitivity of the technique in the subsequent Gm and Gc grouping Protein concentrations approximately equal to those of serum were obtained in only 4 urine concentrates

TABLE 1

Gm and Gc Group Determination of Concentrates of Proteinuric Urine Samples and the Corresponding Sera

Journal No	Serum		Urine Concentrates		
	Gm	Gc	Protein Content per cent	Gm	Gc
B 106/62	a+b+x—	1-1	1 1		
B 1934/61	a—b+x—		1 5		
B 2369/61	a+b+x—	2-1	1 6		
L 1011/61	a+b+x—	1-1	3 1		
B 2254/61	a+b+x—	2 1	abt 4		
B 2261/61	a+b—x+	2 1	abt 4		
B 132/62	a+b+x—	1 1	6 9	a+b+x—	1-1
B 181/62	a—b+x—	2 1	9 2	a—b+x—	2 1
B 102/62	a+b+x+	1 1	10 0	a+b+x+	1 1
B 2041/61	a+b+x—	2 1	12 1	a+b+x—	2 1

The total serum protein values in the donors of the proteinuric urines ranged from 4 7 to 6 9 per cent The fractions of serum protein did not differ from what would be expected in cases of preeclampsia, i e albumin and gamma globulin values moderately decreased, alfa- and beta-globulin values proportionally raised

Results of the Gm Grouping

The few samples of concentrated normal urine did not contain any demonstrable Gm substance The concentrates of proteinuric urines were less inhibiting than the corresponding sera, but they appeared to allow group determination in 4 cases in which the protein contents ranged from 6 9 12 1 per cent An example is shown in Table 2 In the remaining 6 cases group determination was not possible, the degree of inhibition for one or more of the Gm factors being too small to be conclusive

TABLE 2

Gm Group Determination of Urine Concentrate and Serum Originating in Patient no B 132/62 Gm Group Gm(a+b+x—)

Aggl systems	Samples to be examined		Dilution of samples 1 1	1 2	1 4	1 8	1 16	1 32
Gm(a)								
Serum "1604" 1 20 anti D "07"	B 132/62	Urine conc	—	—	—	++	+++	+++
		Serum		—	—	—	++	+++
	Serum Controls	Gm(a+)		—	—	—	+	++
		Gm(a—)		++	+++	+++	++++	++++
Gm(b)								
Serum "Berg" 1 15 anti D "42"	B 132/62	Urine conc	—	—	—	++	++	++
		Serum		—	—	—	++	++
	Serum Controls	Gm(b+)		—	—	—	—	+
		Gm(b—)		++	++	++	++	++
Gm(x)								
Serum "Rolf" 1 40 anti D 07"	B 132/62	Urine conc	++	+++	++++	++++	++++	++++
		Serum		+	+++	++++	++++	++++
	Serum Controls	Gm(x+)		—	—	—	+	+++
		Gm(x—)		++	+++	++++	++++	++++

RESULTS

The samples of normal urine were concentrated approximately 100 times as estimated by control weighings, but protein concentrations detectable by Kjeldahl's nitrogen analysis were not obtained. The samples of proteinuric urine were concentrated 5–50 times. This marked variation was due to differences in the duration of the dialysis against dextran as well as differences in the protein contents of the samples prior to concentration. The final protein contents of the proteinuric urine samples ranged from 1.1–12.1 per cent. This variation in the protein contents made it possible to estimate the sensitivity of the technique in the subsequent Gm and Gc grouping. Protein concentrations approximately equal to those of serum were obtained in only 4 urine concentrates.

TABLE 1

Gm and Gc Group Determination of Concentrates of Proteinuric Urine Samples and the Corresponding Sera

Journal No.	Serum		Urine Concentrates		
	Gm	Gc	Protein Content per cent	Gm	Gc
B 106/62	a+b+x−	1-1	1.1		
B 1934/61	a−b+x−		1.5		
B 2369/61	a+b+x−	2-2	1.6		
L 1011/61	a+b+x−	1-1	3.1		
B 2254/61	a+b+x−	2-1	abt. 4		
B 2261/61	a+b−x+	2-1	abt. 4		
B 132/62	a+b+x−	1-1	6.9	a+b+x−	1-1
B 181/62	a−b+x−	2-1	9.2	a−b+x−	2-1
B 102/62	a+b+x+	1-1	10.0	a+b+x+	1-1
B 2041/61	a+b+x−	2-1	12.1	a+b+x−	2-1

The total serum protein values in the donors of the proteinuric urines ranged from 4.7 to 6.9 per cent. The fractions of serum protein did not differ from what would be expected in cases of preeclampsia, i.e. albumin and gamma globulin values moderately decreased, alfa- and beta-globulin values proportionally raised.

Results of the Gm Grouping

The few samples of concentrated normal urine did not contain any demonstrable Gm substance. The concentrates of proteinuric urines were less inhibiting than the corresponding sera, but they appeared to allow group determination in 4 cases in which the protein contents ranged from 6.9–12.1 per cent. An example is shown in Table 2. In the remaining 6 cases group determination was not possible, the degree of inhibition for one or more of the Gm factors being too small to be conclusive.

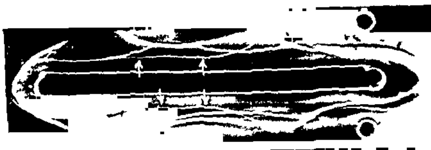


Fig 2

Immunoelectrophoretic pattern illustrating the verification of the identity of a slightly displaced Gc 1 1 precipitate originating in a urine concentrate (B 132/62) by admixture of an equal volume of serum reference of type Gc 2 2 (above) Serum reference of type Gc 2 1 (below) Antiserum B 146

poorly stored serum samples, *viz* a drawing out of the precipitates in the direction of the anode and weakening of the 2-component for instance in samples of Gc type 2 1 In all 3 cases the group determination was verified by repeated analyses after admixture of sera of known Gc types and by absorption tests An example is given in Fig 2

In 4 cases distinct patterns appeared, but individually without any unquestionable Gc arc or with a Gc like precipitate, the identity of which, however, could not be verified In one of these concentrates the absorption test indicated the presence of Gc substance

In the last 2 cases few precipitates were seen, one of which the albumin arc

The employed immune serum did not allow a complete analysis of the protein fractions of the urine concentrates The patterns however displayed in every case distinct albumin and transferrin precipitates Furthermore precipitates appeared in the alpha 1 and alpha 2 region the latter of a certain resemblance to hp and macroglobulin
presented obs
precipitates e
mentioned by
has not been
to urinary sediments in the antigen basin followed by a successive liberation of the protein

The results of the Gm and Gc group determination of the urine concentrates appear from Table 1

DISCUSSION

The present investigation has demonstrated the presence of group specific Gm and Gc substances in concentrated urine samples from pregnant women suffering from preeclampsia, provided the protein contents of the urine concentrates were equal to or higher than 6 9 per cent This finding is concordant with the observation made by others (15 7 18) that proteinuric urine, including urine from patients suf-



Fig 1

Immunoelectrophoretic pattern of a urine concentrate originating in donor B 102/62 showing a typical Gc 1-1 precipitate (A) Admixture of serum of type Gc 1-1 to the urine concentrate does not change the Gc precipitate (C) whereas admixture of serum of type Gc 2-2 produces a Gc 2-1 precipitate (E)

B Serum from the donor B 102/62
D Serum reference of type Gc 1-1
F Serum reference of type Gc 2-2
Antiserum used throughout R 146

Results of the Gc Grouping

Gc grouping of concentrated normal urine was not possible, but normal urine in a single case was able to remove anti-Gc from immune serum by absorption

The immuno-electrophoretic patterns of the concentrates of proteinuric urines allowed Gc grouping in the same 4 cases in which Gm grouping was possible In one of these cases the appearance was very much like that of a normal serum pattern (Fig 1) The Gc arc was indistinguishable from a normal Gc immuno-precipitate of serum belonging to Gc type 1-1 Admixture of serum belonging to this type did not modify the Gc arc of the urine concentrate, whereas the addition of serum belonging to Gc 2-2 produced a clearcut identity reaction In the absorption test all anti-Gc was removed In the other 3 cases the immuno-electrophoretic patterns were slightly altered, as it may be seen in older,

ployed technique, nor may contamination with urine be assumed to interfere with Gm and Gc grouping of blood stains

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fering from the nephrotic syndrome, contains several serum proteins though in varying concentration, including alfa-2-globulins and gamma globulin

As regards the few examined concentrated normal urine samples Gm and Gc group determination was not possible Only in a single case did the absorption test reveal the presence of Gc substance, but in a concentration that did not allow Gc grouping

A probable explanation of this seems to be insufficiency of the employed process of concentration The techniques used for Gm and Gc determination in the present work appear to require a protein concentration of the examined sample approximating that of serum Qualitative and quantitative differences in the proteins of normal and pathological urines may play a certain rôle, but it must be stressed that normal as well as pathological urines contain serum protein fractions including alfa-2 and gamma-globulins (For a comprehensive survey see 18)

From a theoretical point of view the importance for forensic medicine of the presence of Gm and Gc substances in urine seems obvious The extent of the present investigation does not allow any estimation of the practical consequences of this observation However, the results seem to suggest that Gm and Gc grouping of urine stains from normal or even from proteinuric urines will not be possible, on the other hand contamination with urine cannot be assumed to interfere with Gm and Gc grouping of blood stains (14, 13)

SUMMARY

A series of normal and proteinuric human urine samples were examined with regard to the Gm and Gc contents

The samples were concentrated by dialysis against a 10 per cent dextran solution In the Gm group determination, comprising the factors Gm(a), Gm(b) and Gm(x), a titration technique was employed The Gc group determination was performed by immuno electrophoresis

The concentration technique proved inadequate for normal urine The proteinuric urine samples were concentrated to a final protein concentration of 1 1 to 12 1 per cent Gm and Gc group determination was practicable in 4 cases, in which the protein contents of the concentrates ranged from 6 9 to 12 1 per cent No discrepancies between the Gm and Gc types in urine and the corresponding sera were found In the remaining 6 cases, in which Gm and Gc grouping was not practicable, the protein contents of the concentrates were under 5 per cent

The results are compared with previous immuno electrophoretic examinations of urine proteins The extent of the present investigation does not allow any estimation of the consequences for medico-legal practice of the findings However, the results seem to suggest that Gm and Gc grouping of stains of even proteinuric will not be possible by the em-

TRANSACTIONS

OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting December 1–2, 1961

Received 09 ii 63

1 *M. Nasiell*: THE GENERAL APPEARANCE OF THE BRONCHIAL MUCOSA IN BRONCHIAL CANCER

The bronchial mucosa has been investigated in 50 cases of bronchial carcinoma and 56 control cases. Basal cell hyperplasia was often found in both series. A similar alteration ("pretransitional epithelium") and also "slit formation and expulsion" were however more common in the cancer material. A slit is formed underneath the surface cells resulting in a low epithelium without cilia. This probably provides the histopathological explanation for Papanicolaous Ciliocytophthoria. Keratinized squamous epithelium was found in carcinoma cases only and always within the tumour. Papilloma like stromaproliferations ("micropapilloma") had occurred in 60 and 25 per cent respectively of the cases while metaplastic epithelium (formed via basal cell hyperplasia or low epithelium) was found in 80 and 34 per cent respectively. Advanced epithelial atypia and extensive metaplasia was observed only in the cancer series and the former only in connection with transitional metaplasia. This suggested the existence of a generalized epithelial disturbance in the cancer cases.

2 *Th. Berge* and *O. Grøntoft*: CYTOLOGICAL DIAGNOSIS OF PLEURAL MESOTHELIOMA

3 *O. Grøntoft*: HISTO-PATHOLOGICAL DIAGNOSIS OF NEEDLE BIOPSIES FROM THE PROSTATE GLAND (prel. communication)

4 *A. Bäckgren*, *L. E. Johansson*, *A. Nordén*, *S. Rubarth* and *G. Winqvist*: SPONTANEOUS LEUCOSIS IN DOGS

In Sweden leucosis in dogs is not uncommon. For the last 25 years this disease has been found in 2 per cent of the autopsy material in the Department of Pathology of the Royal Veterinary College. The 200 cases of leucosis comprised 179 cases of a lymphatic type, 17 of a myeloid type and 3 of a plasmacell type. The disease was found in dogs ½–13 years of age. The mean age was 6.7 years. The mean age for the whole autopsy material during this period was 4.32 years. Sex distribution of the whole material was 51.5 per cent males and 48.5 per cent females. In the leucosis material 58.8 per cent were males and 41.2 per cent females. The disease seems to be predominant in males, this is however not significant. As carcinoma in dogs leucosis appears most frequently in boxers and in Airedale terriers. In connection with the advanced mean age this perhaps may be an expression for the blastomatous character of leucosis.

BRIEF REPORT

QUANTITATIVE DATA ON THE CELL CONTENT OF THE FEMORAL BONE MARROW IN THE ADULT FEMALE ALBINO RAT

By *F. Bierring* and *I. Grunnet*

The technique adopted in the present investigations differs in only slight detail from that described by *Ramsell & Yoffey* (1961) for quantitative examination of the bone marrow in the rat.

Under ether anaesthesia the animals were killed by bleeding through incision of the abdominal aorta. The blood was collected, centrifuged and the serum pipetted off. Both femurs were removed immediately after the bleeding. They were cleaned, the epiphyses sawn off and the diaphyses immediately carefully split lengthwise. The bone marrow cylinders thus exposed were transferred to a glass tube with a tight fitting rubber stopper containing a previously weighed amount of the autogenous serum sufficient to give a dilution of bone marrow in serum of approximately 1:15. The bone marrow cells were suspended in the serum by vigorous mechanical shaking for three minutes. The suspension was weighed and the specific gravity of the suspension and the serum was determined by the copper sulphate method. The volume of suspension and of serum was calculated and from these values the volume of bone marrow used was determined. The volume dilution of bone marrow in serum could then be calculated. The absolute number of nucleated cells per mm^3 of suspension—counted in a haemocytometer after dilution (1:40) with a solution of methyl violet and acetic acid—was converted into the number per mm^3 of bone marrow by means of the volume dilution. The absolute number of erythrocytes per mm^3 of bone marrow was determined in the same way, Hayem's fluid being used as a diluent. Smears of the suspension were prepared using the serum film technique of *Harris* (1956) and the smears were stained by the May-Grunwald-Giemsa method. Differential counts were made on 2000 cells from each suspension and the absolute number of cells per mm^3 of bone marrow calculated for each main cell group.

The following are the mean values obtained for the total number of nucleated cells and for the number of cells in the main cell groups per mm^3 of bone marrow in 10 normal rats (all figures including the standard deviation $\times 10^3$).

The total number of nucleated cells amounted to 2208.9 ± 236.0 cells. The absolute numbers of cells in the main cell groups were: *Lymphocytes* 410.0 ± 81.2. The majority of these cells were small lymphocytes but lymphocytes of all sizes are included in this group. *Erythroid cells*, i.e. all nucleated precursors of the erythrocytes, 509.2 ± 101.0. *Myeloid cells*, including mature granulocytes and their pre-

unclassifiable within the main cell groups owing to severe damage.

The data here presented form the basis of experiments the purpose of which is to study the origin of the large population of lymphocytes found in the rat bone marrow. A report on these experiments will appear later. In the present study lymphocytes accounted for 18.8 ± 3.7 per cent of the total number of nucleated cells. As a result of the previous exsanguination the blood content of the bone marrow was slight. The content of erythrocytes in the bone marrow averaged 0.9×10^6 cells per mm^3. The number of erythrocytes per mm^3 of blood in the animals used was 7.7×10^6. Consequently the blood contamination was about 0.117 mm^3 of blood per mm^3 of bone marrow. The lymphocyte content of the blood in these rats averaged 18.6×10^3 per mm^3. Thus only an insignificant portion of the bone marrow lymphocytes owe their presence to the blood content.

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(Comparative Endocrinology Wiley New York 1959) who stated that total pancreatectomy produces a mild type of diabetes in rabbits

The pancreatic rests in the subtotally depancreatized diabetic rabbits have been studied Pictures suggestive but not convincing of so called acino insular transformation have been obtained Glycogen infiltration in hydropic β-cells has been shown

8 S *Falkmer* and F *Knutson* IS COBALT CONCENTRATED IN PANCREATIC ISLET TISSUE?

9 I *Zamboni* ELECTRON MICROSCOPICAL INVESTIGATION ON THE EARLY STAGES OF POLYOMA VIRUS INFECTION IN MICE

A pellet obtained by ultracentrifugation of medium from polyoma infected tissue culture and very rich in virus particles mostly attached to fragments of tissue culture cells was resuspended in a small quantity of fluid and subcutaneously injected in adult mice

15 minutes 45 minutes and 24 hours later subcutaneous tissue was excised from the injection area and studied with the Electron Microscope 15 minutes after the injection virus particles were observed in the cytoplasm of the tissue cells They had been transported through the cell membrane as single units by pinocytotic activity or in clusters attached to the injected cell fragments by phagocytosis

After 45 minutes few particles were detectable intranuclearly whereas 24 hours later the nuclei of many cells were litterally loaded with virus The degeneration of the infected cells leads to a fragmentation of the elements their debris rich in virus particles are phagocyted by healthy cells with consequential spreading of the infection

10 A *Bergstrand* C G *Bergstrand* N *Engstrom* and K M *Herrlin* RENAL CHANGES DURING TRIDIONE TREATMENT

A nephrotic syndrome is a rare complication to treatment of epilepsy in children with Tridione®. Experimental investigations also indicate that Tridione in large doses may have a nephrotoxic effect In order to find out if slight and clinically non observed damage to the kidneys is a frequent complication we have investigated seven cases aged 15 17 years which have been treated with Tridione for periods between three months and eights years Continuous renal examination showed only slight and transient haematuria or proteinuria in three of the cases

Light microscopy of renal needle biopsy material showed no glomerular or tubular lesions

Electron microscopy of the glomeruli showed wide variations in the appearance of the capillary epithelial cells These are regarded as physiological variations and not as pathological phenomena

The investigation has not shwon any signs of renal damage which could be assigned to the treatment

11 B *Thorell* LOCALIZATION AND DETERMINATION OF RESPIRATORY ENZYMES IN THE SINGLE LIVING CELL

With regard to the fundamental energy linked cell reactions e g the cell respiration our knowledge about the functions of the various cell structures has

5 *B Lagerlof* TRANSPLANTATION OF CHROMOSOME LABELLED LEUKAEMIA CELLS

A transplantation form of virus induced erythroleukaemia in the fowl can be obtained by inoculating the chick with intact leukaemia cells. The host's own bone marrow cells, which are the target for the virus may, however, be infected by the virus released from the leukaemia cells and thereby undergo leukaemic transformation. In its final stage the disease thus developed would then be a combination of transplantation- and virus induced leukaemia.

The occurrence of transplanted and autochthonous leukaemia cells in the body has been studied by chromosome analysis. Hens, the cells of which have 11 macro-chromosomes and an unpaired fifth chromosome, were inoculated with leukaemia cells of cocks which have 12 macro chromosomes and paired fifth chromosomes. Three and four days after such inoculation the animals showed varying degrees of transplanted leukaemic proliferation in the bone marrow and in the spleens the infiltration was in most cases due entirely to the proliferation of the transplanted cells. On the fifth day a few of the host's own cells were also recognized in the spleen and the bone marrow contained a mixture of autochthonous and transplanted leukaemic proliferation. It was concluded that during the first days the animals can develop a fairly pure transplantation leukaemia on which a virus induced component is subsequently superimposed.

6 *G Hultquist* and *J Thorell* TRIALS WITH PANCREAS TRANSPLANTATION IN RATS AND GUINEA-PIGS

Pancreas tissue from rats or guinea pigs with pancreatic duct ligation and containing islet tissue but no other remains of exocrinic parenchyma except ducts was implanted in the anterior chamber of the eye. The transplantations were carried out both in the animals with duct ligation and in other members of the same litter. After one month islet tissue showing signs of growth was found in many of the rats, about 90 per cent of the case of autologous transplantation and about 35 per cent of the others. Much poorer results were shown by the guinea pigs in which the survival time was 9-10 days at the maximum.

The grafts always contained β cells showing a pseudo isocyanine reaction but α cells were present only in some cases. In a few α cells a positive reaction was obtained with Voigt's sulfide silver method.

Karyometry showed nuclear enlargement in both β and α cells with maximum enlargement in 3-4 day grafts. The change gradually decreased and after 3-4 weeks the values corresponded to those of the normal pancreas.

7 *G Nathorst Windahl* DIABETES IN PANCREATECTOMIZED RABBITS

Subtotal pancreatectomy in rabbits produces a relatively mild type of diabetes with glycosuria polyuria hyperglycaemia and an initial loss of weight. There is often an overproduction of ketone bodies but severe acidosis or coma does not develop. The animals are able to live for long periods without insulin and yet often maintain an almost constant rather low body weight in spite of pronounced glycosuria.

Total or approximately total pancreastctomy however produces a severe diabetes with massive ketonaemia and ketonuria. Metabolic acidosis and coma develop in a few days unless insulin is given.

These results are at variance with the opinion of Greeley as cited by Houssay

acid fast Pollen was suspended in physiological saline and injected subcutaneously and intraperitoneally into rabbits (33 animals) The animals were killed 1–8 months later Foreign body granulomas developed around the injections but no epithelioid cell granulomatosis was observed The experiments provided no support to the hypothesis of a causal relationship between pollen and sarcoidosis

18 *L E Larsson* ENDOCARDIAL MYXOMA WITH MALIGNANT DEGENERATION

A woman without known rheumatic fever had for 3 years had progressive cardiac failure clinically interpreted as combined mitral and aortic disease During the last few months widespread skeletal lesions of malignant character were observed Necropsy revealed a myxomatous tumour at the fossa ovalis in the left atrium The endocardium in the atrium was thickened the mitral orifice was stenosed the mitral valves and chordae tendinae were rigid and thick and the aortic valves showed diffuse fibrous thickening The left lung showed a hilar tumourous nodule with peripheral extensions along the vessels Numerous metastases were seen in the heart wall in the skeleton and in the left ovary The atrial tumour exhibited the histological characteristics of an endocardial myxoma with sarcomatous degeneration The rest of the atrial wall the mitral and aortic valves chordae tendinae and the pulmonary veins showed marked fibrous thickening with a surface layer of *anaplastic tumour cells The relations between the tumour and the valvular and* pulmonary lesions are discussed

19 *L Olding* PERINATAL BACTERIAL INFECTIONS IN AN AUTOPSY MATERIAL

20 *C Lundmark* GRANULAR ATROPHY OF THE BRAIN—OBLITERATING ARTERIAL DISEASE

21 *C Lundmark* CARCINOID TUMOUR WITH METASTASES IN THE HEART AND AORTIC STENOSIS

22 *B Larsson* A CASE OF POSTANGIOGRAPHIC Th DEPOSITION AND PRIMARY SQUAMOUS-CELL CARCINOMA OF THE LIVER

A bilateral cerebral angiography had been performed with a total of 17 ml of Thorotrast Twenty seven years later the 53 year old male succombed to a large necrotizing liver tumour squamous-cell in type Au^{198} total body scintillography had shown no uptake in the region of the spleen before [illegible]

[illegible] g and revealed a moderate and mainly post necrotic scarring Thorotrast deposition was demonstrated in liver and spleen with routine methods and autoradiography The bone marrow was in a reactive state A moderate anaemia was present There were no oesophageal varices

so far mainly been based on bulk analyses of isaloted tissue fractions The present paper describes some recent developments of the microspectrographic technique which permit quantitative *in vivo* assay of respiratory enzymes in localized areas of 1 5 μ in diameter in the single intact cell The detectability is equivalent to 6000 molecules of cytochrome *b* Soret spectra of both oxidised and reduced cytochromes can be demonstrated depending on whether the cell is in an aerobic or an anaerobic state Measurements on the grasshopper spermatocyte and on isolated liver and kidney cells have given information about the metabolic state of the respiratory system even of the single structures within the living cell

12 *K F Aström* EXPERIMENTS ON ALLERGIC ENCEPHALOMYELITIS

13 *S Szögi* ASEPTIC MENINGOENCEPHALITIS AND MYOCARDITIS (PROBABLY COCKSACKIE) IN A NEW BORN CHILD

14 *S Lundberg and O Grontoft* *A CASE OF BILATERAL SYMMETRICAL CALCIFICATIONS OF THE BRAIN*

15 *I Bjersing* and *N F Borglin* FOSINOPHILIA IN UTERUS

In recent years the authors have occasionally seen massive collections of eosinophilic leucocytes in the human uterine myometrium Fifteen cases were studied in detail Two groups were used for comparison In one of the groups amputation of the uterus or cervix had been preceded by curettage or biopsy but not in the other Eosinophilic granulocytes were observed—also in the controls—usually around vessels or between muscle bundles in the myometrium and showed no correlation with the amout or site of other inflammatory cells No explanation can be offered for this abnormal eosinophilia which has as far as we know not been described before The eosinophilia was slight and it was rare when the interval between the interventions was more than 3 weeks Animal experiments in progress have shown absence of eosinophilic leucocytes in the uterus of spayed rats and eosinophilia in spayed animals treated with oestrogen the degree of eosinophilia varying closely with the dose given

16 *G Fichera* and *I Hagerstrand* LYMPHATIC CHANGES IN CHRONIC PULMONARY CONGESTION

A post mortem series of chronic pulmonary congestion (100 cases of mitral stenosis 75 cases of pulmonary congestion because of hypertension or myocardial lesions) was studied Compared with the controls (healthy lungs from 50 cases of different ages) fibrous mural thickening of the lymphatics was *found at different* levels (perivascular peribronchial interlobular and intrapleural) in 70 per cent of the cases of pulmonary congestion This lymphatic fibrosis appeared to develop together with other pathological signs of chronic congestion and was probably not a primary lesion *The fibrosis was probably due to increased drainage* of fluid

17 *I Hagerstrand* and *F Linell* SARCOIDOSIS AND POLLEN

Experiments were performed to check the hypothesis put forward in recent years that sarcoidosis might be due to fir tree pollen All of the pollen studied (different sorts of fir tree pine beech oak maple alder lycopodium timothy grass) proved

posterior diencephalic lesions However these results are preliminary and must be confirmed by further studies

The results support the view that there is no localized "sexual center" in the adult male rabbit However posterior diencephalic lesions seem to induce alterations in spermatogenesis

2 *P Sourander* MORBUS KRABBE

4 *I Hagerstrand* THALIDOMID INDUCED MALFORMATIONS

5 *H Sunzel* and *L Zettergren* LESIONS IN THE LIVER AFTER SURGICAL OPERATIONS IN THE UPPER PART OF THE ABDOMEN

6 *L Enerback* HISTO CHEMICAL REACTIONS IN CARCINOIDS

7 *F Knutson* AUTORADIOGRAPHY OF PITUITARY CELLS IN ADRENALECTOMIZED WHITE RATS AFTER THE INJECTION OF ADENINE-14C

Five male white rats weighing 150–200 gramts were adrenalectomized Observation times were 1 2 4 8 and 12 days Two hours before the end of each observation time the animals were injected with 0 05 mC adenine 14C.

The pituitaries were fixed in neutral formalin embedded in paraffin and sectioned serially Autoradiography was performed with strip film technique and Kodak AR 10 emulsion The unstained autoradiographic specimen was mounted in a glycerol medium and examined under a photo microscope with a micron graduated mechanical stage Labelled cells were photographed in transmitted light and in phase contrast The the film was removed and the section stained with Tri PAS technique With the aid of the graduated stage and the previously taken photographs the same labelled cells were localized in the stained section

About 70 labelled cells within random fields in all pituitaries had been so localized One day after adrenalectomy 70 per cent of labelled cells were small chromophobes With increasing time after adrenalectomy their percentage gradually diminished whereas the number of labelled large vacuolized chromophobes and amphophiles increased reaching almost 50 per cent 8 and 12 days after adrenalectomy Very few well granulated basophiles were labelled The results point at the chromophobe cell as the site of new production of ATCH and it was suggested that these chromophobes gradually turned into vacuolized forms and so called amphophiles

8 *P Lundin* THE CARCINOGENIC ACTION OF COMPLEX IRON PREPARATIONS

9 *H Röckert* and *L Zettergren* LESIONS CAUSED BY BARIUM CONTRAST MEDIA

10 *I Angervall* and *S E Fagerberg* SKIN BIOPSY IN DIABETES

Since the beginning of 1961 punch biopsies from the skin on the dorsal part of the basal phalanx of the 4th toe of diabetics have been examined All specimens have been fixed in 10 per cent neutral formalin solution about half of them being

TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting April 7, 1962

1 *I.-B. Schnürer* and *S. Stattin*: THE VASCULAR SUPPLY OF TENTORIAL MENINGIOMAS

In some papers dealing with tentorial meningiomas vessels have been described seemingly arising from the carotid siphon. This prompted an investigation on the topography and distribution of these vessels performed on autopsy specimens with the aid of a dissection microscope. The vessels were successfully injected and dissected in about 15 of the specimens. The number and course of the major branches were found to be rather constant. Thus there are two main branches, one arising from the infero-lateral aspect of the so called horizontal portion of the siphon, the other from the upper posterior aspect of the convexity of the proximal portion of the siphon. In most cases both main stems give off tentorial branches, in some only one of them does. The tentorial branch of the lateral main stem in most cases runs directly upward, enters the tentorium and continues backward near the free margin, sometimes up to the summit. The tentorial branch of the dorsal main stem is usually smaller and runs backward in the tentorium near its attachment to the petrous bone. These tentorial vessels are most often but not always too small to be visualized at angiography but they may hypertrophy when contributing to the vascular supply of mengiomas and vascular malformations and thus be of angiographic and neurosurgical importance.

2 *C. Ahren*: EFFECT OF DIENCEPHALIC LESIONS ON TESTES IN ADULT RABBITS

Diencephalic lesions were produced in 248 adult male rabbits with the aid of Ranstrom's X-ray stereotaxic instrument in medial parts of the diencephalon from the anterior medial preoptic area to the rostral border of the mesencephalon. The animals were allowed to survive for 6 weeks, half of these were subjected to repeated subcutaneous injections of formalin during the final 3 weeks. On the basis of the size, localization and symmetry of the lesions determined histologically from serial sections of the diencephalon 140 animals were segregated into 10 groups (Each about equally devided as to formalin treatment.) The median eminence and pituitary gland were histologically intact in all animals.

Results: Some animals showed gross testicular atrophy not correlated to any particular lesion. Severe atrophy was only observed with formalin treatment, probably a non specific stress reaction. There were no significant differences in the mean testicular weight in the 10 groups as compared with the control animals. Histological signs of altered spermatogenesis in some animals suggest disturbances in secretion of FSH. Such changes were most frequently observed in animals with

TRANSACTIONS
OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting December 1, 1962

1 L Santesson ON THE CLASSIFICATION OF OVARIAN TUMOURS

At an international conference in Stockholm the following proposal was made
The Conference recommends that the common epithelial tumours of the ovary be classified in the following manner

1 *Serous cystomas*
   a) Serous papillary cystadenoma benign (Figs 69 and 71)
   b) Proliferating serous papillary cystadenoma without stromal invasion (possibly malignant) (Figs 72 and 75)
   c) Serous cystadenocarcinoma all grades (Figs 78 and 81)
2 *Mucinous cystomas*
   a) Mucinous cystadenoma benign (Figs 87 and 90)
   b) Proliferating mucinous cystadenoma without stromal invasion(possibly mal gnant) (Figs 91 and 92)
   c) Mucinous cystadenocarcinoma all grades (Figs 93 and 95)
3 *Endometrioid tumours*
   a) [1]
   b) Prol ferating endometrioid adenoma and cystadenoma (possibly malignant) (Fig 102)
   c) Endometrioid adenocarcinoma all grades (Figs 77 and 105)
4 *Undifferentiated carcinoma* (cell type unknown)

[The numbers in parenthesis refer to figures published in 'Tumors of the Ovary and Fallopian Tube by *Hertig A T & Gore H* (Atlas of Tumor Pathology Section IX Fascicle 33 Tumors of the Female Sex Organs Part 3 Armed Forces Institute of Pathology Washington 1961)]

Usually in any given tumour only one type of epithelial cell is dominating Occasionally when there is a mixture of different cell types the predominant type should determ ne the diagnosis

2 *S Falkmer F Knutson* and *G E Voigt* FURTHER STUDIES ON THE COBALT CONCENTRATING ABILITY OF PANCREATIC ISLET TISSUE

The recent discovery by quantitative scintillation determinations using ^{58}Co that isolated islet tissue has approximately the same high ability as the liver to con

[1] The existence of a blank space after 3 a) is due to the fact that there is as yet no agreement as to whether some cases of ovarian endometriosis may properly be classed as true tum urs or all must be regarded as simply displaced or ectopic endometrium

fixed also in 4 per cent basic lead acetate solution The skin specimens were cut into 4 μ serial sections consecutive sections being stained with haematoxylin eosine van Gieson haematoxylin Hansen and MacManus' periodic acid Schiff So far we have examined biopsy specimens from 50 male and female diabetics of varying ages and from 35 non diabetics

All but one of the diabetics exhibited dermal microangiopathy in the form of wall thickening and reduction in the lumen due to deposition of a strongly PAS positive substance which in van Gieson stained sections was a homogeneous mildly picrinophilic materal Some vessels displayed hypertrophic hyperchromatic nuclei between PAS positive fibrils of the basement membrane The diabetic microangiopathy is usually most pronounced in the capillaries surrounding the sweat glands and in the papillae The larger the vessels the more difficult it is to find characteristic lesions

All but one of the controls showed in general a suggested or slight PAS positive reaction

The facts that diabetic microangiopathy has now been encountered in the skin constitutes additional evidence that it occurs throughout the organism

chicks with a late onset of the disease dying at an age of more than one month had a significant increase of the gamma globulin fraction This probably contains viral antibodies and may thus be responsible for the fact that the virus concentration of the plasma from leukaemic chicks decreases with increasing age of the birds Young chicks with marked viraemia had normal amounts of gamma globulin Chicks even young ones with the subleukaemic anaemia had high gamma globulin fractions which taken together with the lymphoid hyperplasia and the signs of haemolysis seems to indicate an immunologic cause of the disease

6 *T Saldeen* EXPERIMENTAL INVESTIGATION OF CANCER SPREAD TO THE LIVER IN RATS WITH LIVER CIRRHOSIS

Moderate liver cirrhosis induced by administration of carbon tetrachloride to rats increased the percentage of secondary intrahepatic growths following intraportal injection of tumour cells from Rous rat sarcoma The number of tumour nodules in the liver was higher when the interval between the last injection of carbon tetrachloride and the injection of tumour cells was 4 or 6 weeks than when it was 2 weeks A positive correlation was found between the extent of fibrosis in the liver and the number of tumour nodules

After injection of tumour cells into a tail vein tumour nodules were seen in most cirrhotic but in no normal livers

Liver cirrhosis had no effect on the growth of the tumour after direct intrahepatic injection of the tumour cells suggesting that the vascular changes in the liver are responsible for the increased number of intrahepatic secondary growths

A possible increase in the number of tumour cells trapped in the cirrhotic livers can however hardly explain the higher percentage of takes in the liver Another possibility is that the narrowing and obliteration of the smaller vessels prevented the tumour cells from coming into contact with Kupffers cells

7 *J Zajicek* NEEDLE BIOPSIES AS A METHOD TO OBTAIN HUMAN TUMOUR MATERIAL FOR BIOCHEMICAL AND BIOLOGICAL INVESTIGATIONS

8 *P S Persson* CYTOLOGIC DIAGNOSIS OF SUBACUTE AND CHRONIC THYROIDITIS

Thin needle punctures were carried out in 175 patients with goitres The smears were stained according to the May Grunewald Giemsa method Of 9 patients with the clinical picture of subacute thyroiditis 6 had the cytologic findings of subacute thyroiditis and 2 a suspected one No definite diagnosis could be made in 1 patient In 42 patients the cytologic diagnosis was chronic thyroiditis In 24 patients the diagnosis was verified by histopathological examination the rest had a typical clinical picture There were no cytologic differences between various kinds of chronic thyroiditis

The cytologic findings of subacute thyroiditis vary with the phase of the disease and present the following features (1) marked degeneration of the follicular epithelial cells (2) proliferation of the follicular epithelial cells in the late phase of the disease (3) foreign body giant cells (4) collections of polymorphonuclear leucocytes histiocytes and mononuclear inflammatory cells

The cytology of chronic thyroiditis is characterized by (1) proliferation and to a lower degree (2) degeneration of the follicular epithelial cells (3) collections

centrate cobalt (Acta Endocr 42 1963) prompted us to try further localization of the cobalt in the pancreatic islets As conventional autoradiography had shown that the accumulated cobalt was easily dissolved from the tissue during fixation and embedding procedures we had to modify the histochemical and autoradiographical methods

The isolated islet tissue of the marine teleost *Cottus scorpius* was cut in a microtome cryostat Autoradiography was performed without the sections coming in contact with water Other islets were gassed with hydrogen sulphide, sectioned in the microtome cryostat and subjected to a modified sulphide silver procedure to detect precipitated cobaltous sulphide after extracting other heavy metal sulphides The injected cobalt was localized to the islet parenchyma and not to the stroma By comparing the autoradiograms and the sulphide silver sections with adjacent sections stained with aldehyde fuchsin (for the visualisation of the B and A cells) it was possible to show that the injected cobalt was confined only to the cells in the dark central region of the sculpin islets containing the B cells and the argyrophil A cells

3 *B Stenkvist* GROWTH CURVES, HISTOLOGICAL APPEARANCE AND VIRAL TITRES OF ROUS SARCOMAS A COMPARISON BETWEEN PROGRESSIVE AND REGRESSIVE GROWING TUMOURS

4 *J Thorell* THE DIAPLACENTAL TRANSFER OF INSULIN ANTIBODIES

The insulin antibody titre was assayed by measuring the capacity of serum to bind insulin-I^{131} After incubating the sera with insulin-I^{131} for 5 days at 4° C the antibody-bound insulin was separated from the unbound insulin by paper electrophoresis according to *Berson & Yalow* The antibody titres were assayed in blood obtained from the umbilical cord and from the mother on the same occasion

In pregnant guinea pigs with relatively low insulin antibody titres the insulin binding capacity of the foetal blood was usually double that of the maternal blood at term

The titres of three newborn children of insulin trated diabetic mothers were about equal to their mothers titres

This placental transfer of antibodies may be of some importance for the foetal insulin metabolism Further studies on this subject are in progress

5 *P Sundelin* and *B Lagerlof* DISEASES INDUCED BY MYELOID LEUKAEMIA VIRUS

Myeloid leukaemia virus can induce several different diseases in the fowl The type of disease induced has been shown earlier to depend on the age of the bird and the amount of virus inoculated An investigation into the pathogenic effect of the myeloid leukaemia virus revealed that our virus strain has a pathogenic spectrum similar to that described earlier and induces myeloid leukaemia visceral lymphomatosis and kidney tumours However in our material an additional form of leukaemia a subleukaemic anaemia was observed This form of the disease is characterized by lymphoid hyperplasia and occasional areas of necrosis in the bone marrow a severe anaemia a comparatively low number of leukaemic cells in the peripheral blood and siderosis of the spleen indicating haemolysis

An electrophoretic investigation of the plasma from leukaemic birds revealed that

11 *A Cederberg* and *G Östberg* OXYGEN INTOXICATION

A girl of eighteen after some days of sore throat suddenly developed pain in the right side of the chest and heavy respiratory distress On the third day she was taken to hospital where blood was found in the right pleura She was given transfusions and treated with drainage of pleura later on tracheotomy and artificial respiration with increasing partiel pressure of oxygen up to 52 per cent in the last hours She died on the tenth day Clinical diagnosis Spontaneous haemo pneumo thorax

At autopsy the lungs which weighed 830 and 870 grams were strikingly heavy and firm No rupture or bleeding was found on the lung surface Microscopically a dense fibrinous exudate filling the alveoli of right lower lobe was seen Other parts showed widespread formation of hyaline membranes on alveolar septa

Pulmonary hyaline membranes of adults are rare They seem to occur without any certain relation to other diseases The rôle of oxygen treatment has been discussed Possibly hyaline membrane formation can be induced by thoracic surgery Our case might have a similar mechanism with collapse of the right lung and compression of the left at spontaneous haemo pneumothorax Delay of treatment may have played a rôle

12 *J Söderstrom* SPLENIC RUPTURE IN INFECTIOUS MONONUCLEOSIS

About 50 cases of splenic rupture in infectious mononucleosis are reported The histological picture is described on the basis of a personal collection of 6 cases Three of the 6 patients had died and 3 had been operated upon with success The spleen showed intense proliferation of large mononuclear cells above all in the red marrow but also in the capsule and the trabecular network that had thereby become loose which probably facilitated the rupture The cellular proliferation in the vessels was noteworthy Large round cells were seen in the adventitia of the arteries and subintimally in the veins Other organs showed similar changes The picture was the same in all the cases studied and coincided with descriptions of earlier reported cases

The most important disease to be considered in the differential diagnosis is leucosis Careful pathological examination of the characteristic—though not pathognomonic—histological picture together with clinical and laboratory data enable a firm diagnosis of mononucleosis infectiosa

13 *A Rausing* GLIOMA INDUCED BY FOREIGN BODY

A 55 year old woman with bilateral symptoms of parkinsonism since 1951 1956 pallidotomy right side leaving two spirals of tantal in the brain 1959 left sided thalamotomy no foreign bodies left behind No relapse on left side speedy recurrence on right side and progressing symptoms of increased intracranial pressure until death in 1962

Autopsy small-orange sized cyst in right sided field of operation Two metal spirals were found in its wall Walnut sized tumour in the right occipital lobe Brownish discoloration in the left sided field of operation *Histology* tissue from right sided cyst wall and tumour and left sided lesion showing malignant glioma No continuity proved between the different sites but made probable by strongly reactive picture in the tissues in between

A growth with this histological picture must have arisen after the first operation *Oppenheimer et al* have succeded in creating spindle-cell sarcomas by subcutaneous

of lymphocytes lymphoblasts plasma cells reticulo endothelial stem cells and phagocytes Epitheloid cells may sometimes be seen

Difficulties in the differential diagnosis exist between thyroiditis and special types of thyrotoxicosis and malignant thyroid diseases

9 *L G Wiman* SULPHYDRYL CHEMISTRY AND FLUORESCENCE MICROSCOPY IN EXFOLIATIVE PULMONARY CYTOLOGY

Two new cytochemical staining procedures for exfoliative cytology are introduced With one method first published in 1959 by the author a high concentration of proteinbound sulphydryl groups is demonstrated in exfoliated tumour cells In ordinary light microscopy these cells stain intensive blue and can be easily distinguished from surrounding normal cells which appear as pale shadows With a second method (1962) the ability of tetracycline to fluoresce in ultraviolet light is used to display tumour cells In a patient treated with tetracycline the substance is concentrated in tumours especially According to their conspicuous yellow green fluorescence exfoliated malignant cells can be easily identified in smears of sputum bronchial secretions aspirates from pulmonary tumours and pleural fluids If unfixed smears from patients not treated with tetracycline are immersed in a tetracycline solution a strong yellow fluorescence is induced in the tumour cells *Upon the same* material Bertalanffy s method was tried using fluorescence microscopy after staining with acridine orange Hereby certain malignant cells got a bright red fluorescence on account of a high content of RNA in cytoplasm and nucleolus which makes them easy to find during screening work However malignant cells exfoliated from a highly differentiated squamous cell carcinoma often have a low content of RNA For this reason they get a faint olive green or orange red fluorescence and may therefore be overlooked in the microscope This and some practical disadvantages *gives the method a limited value in diagnostic cytology The results of all methods* above has been correlated to and compared with the Papanicolaou staining procedure

10 *K Gydell I Juhlin* and *J G Norden* NOCARDIOSIS

A 41 year old female acquired an auto immunologic haemolytic anaemia and was treated with increasing doses of steroids (average dose during 1 year 29 mg of Deltacortril® per day) After 6 months she got fever and chest roentgen showed infiltrations which later cavitated No acid fast bacilli were found guinea pig inoculation was negative but nocardia was cultivated from bronchial secretion After heavy medication with penicillin the patient improved transiently but then she became worse with detoriation and epileptiform seizures Increasing sputum blocked the air passage On treatment with Elkosin® (sulphaisodimidin) the chest roentgen picture became almost normal and nocardia disappeared from the trachea But the blood values decreased and finally she became unconscious and died

Autopsy In the lungs solid yellow foci and a system of cavities were noted The kidneys showed several yellow streaks and a larger necrotic focus which continued into a perirenal abscess with greenish thick necrotic material Such abscesses were also found in all parts of the brain and in musculature (neck and leg) Nocardia could be demonstrated in cultures with material from brain and kidney but in no other organ

The changes of the oral mucosa were the same as in earlier reported cases. The polydysplastic case showed a peculiar discharge of well defined parts of connective tissue demarcated by ingrowing epithelial strands.

In the tooth germs heavy disturbances in the enamel formation were found such as metaplasia of the enamel epithelium with many epithelial whorls. In micro radiograms these appeared as globular irregularly mineralized structures. The enamel was considerably thinner than normal. In the case where the teeth had erupted they showed severe hypoplasias similar to those clinically seen in amelogenesis imperfecta. In all the cases the tooth germs showed *the same disturbances*. It is concluded that amelogenesis imperfecta may form a stigma in the reported forms of epidermolysis bullosa hereditaria.

18. *B. Larsson*: ON MALIGNANT LIVER AND BILE DUCT TUMOURS IN CASES EXPOSED TO THOROTRAST AND AMONG WINEGROWERS WITH LONG-STANDING EXPOSITION TO ARSENIC

The two groups weere briefly presented as available in the litterature. The non epithelial tumours were mainly discussed, carcinomas being further referred to elsewhere. Some differences between the radioactive ThO and the chemically toxic As O were mentioned. The Th sarcomas appeared after an average time of 16 years and the As tumours after 20 years. The Tumour Development Time (TDT) in these human series represents Tomour Deaht Time, in experimental series a Tumour Detectable Time might be registered.

It is obvious that different substances can induce similar tissue responses. In the present *two* sarcoma groups there is *one* cellular main response and *one* terminal lesion.

Human tumours possibly exogenously induced and certain characteristics in the response of some animal species to carcinogenic influence were considered to present a tentative basis for a further study of common denominators in the process of malignant development.

19. *S. Dahlgren*: RENAL CELL CHANGES AFTER INFUSION OF LOW VISCOSITY DEXTRAN

During the last year a great number of cases with changes in the renal tubules of the type known as osmotic nephrosis have been found at post mortems at the Karolinska sjukhuset. 31 cases featuring such changes have been diagnosed and out of these 27 had been given so calleed low viscosity dextran (average molecular weight 40000) in amounts ranging from 100 to 4400 cc. The other four cases had been given rather large doses of various hexosis. It has not been possible to determine whether the tubular changes are accompanied by disturbances of function. In spite of the fact that the commonly used type of dextran [illegible]

[illegible] low viscosity dextran seems striking.

20. *S. Sögi*: ISOLATED TOXOPLASMA MYOCARDITIS IN A CHILD

[illegible]
[illegible]
beer [illegible]

implantation of tantal plates It is suggested that neuroglia may give a malignant response to a similar stimulus

14 *C Lundmark* A FATAL CASE OF VARICELLA

15 *I Olding* EPIDERMOLYSIS BULLOSA LETHALIS SOME MORPHOLOGICAL OBSERVATIONS

Sections of the skin from four infants who all died within nine weeks after birth and who had all been affected with epidermolysis bullosa lethalis were examined by conventional histological methods Sections were stained with haematoxylin and eosin and Weigert's elastin stain The argyrophile reticulum fibers were visualized by Laidlaw's method Sections of skin from dead infants at different ages and without skin diseases were used as controls

The early changes of epidermolysis bullosa lethalis were multiple intracellular vesicles in the basal layer of the epidermis The subepidermal elastin plexus was practically absent but this was also the case in the controls This coincides with the observations of *Dick* (J Anat *81* 201 1947) who has shown that the subepidermal elastin plexus is practically absent in the normal skin in infancy On the other hand the reticulum fibers in the uppermost dermis were well developed both in the cases of epidermolysis bullosa lethalis and in the controls These reticulum fibers were prominent also at the margins of recent bullae where the basal layer showed a marked vesicular degeneration

16 *B Robertson* REPARATIVE PHENOMENA IN "HYALINE MEMBRANE DISEASE"

In an autopsy material of 117 neonatal deaths varying in age between 6 hours and 6 days all displaying pulmonary hyaline membranes proliferative and reparative phenomena in the alveolar epithelium were observed in cases surviving more than two days This agrees with the observations recently reported by *Boss & Craig* (Pediatrics 29 890 1962) Similar changes in the alveolar epithelium were also observed in four other autopsy cases prematures varying in age between 10 and 24 days The clinical picture in these four cases had been that of "idiopathic respiratory distress" since shortly after birth Histological sections from the lungs displayed thickened alveolar walls containing fibroblast like cells and excess of collagen and reticulin No hyaline membranes of the usual type were obeserved in these sections although small amounts of hyaline material were present in some alveoli

These findings reflect a process of repair which indicates previous damage to the alveolar walls possibly connected with the development of hyaline membranes But since the author has also observed similar changes in the alveolar walls of 2 3 week old neonates without typical clinical history of "idiopathic respiratory distress" it is too early to draw any definite conclusions A clinical analysis of these cases is in progress

17 *T Arwill A Bergenholtz* and *O Olsson* EPIDERMOLYSIS BULLOSA HEREDITARIA A HISTOPATHOLOGICAL STUDY OF TEETH AND ORAL MUCOSA IN THREE CASES

Demineralized sections and microradiographs in ground sections from the jaws of patients with the dysplastic and letalis forms of e b h were studied

The changes of the oral mucosa were the same as in earlier reported cases. The polydysplastic case showed a peculiar discharge of well defined parts of connective tissue demarcated by ingrowing epithelial strands.

In the tooth germs heavy disturbances in the enamel formation were found such as metaplasia of the enamel epithelium with many epithelial whorls. In microradiograms these appeared as globular irregularly mineralized structures. The enamel was considerably thinner than normal. In the case where the teeth had erupted they showed severe hypoplasias similar to those clinically seen in amelogenesis imperfecta. In all the cases the tooth germs showed the same disturbances. It is concluded that amelogenesis imperfecta may form a stigma in the reported forms of epidermolysis bullosa hereditaria.

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20. *S. Szögi*: ISOLATED TOXOPLASMA MYOCARDITIS IN A CHILD

Necropsy report of a 5 year-old boy, who was found dead in his bed. The boy had often had infections of the respiratory tract. During the previous 6 months he had been fairly depressed.

The only gross finding of interest at autopsy was enlargement of the heart (wt 120 gm) Microscopic examination however revealed focal myocarditis with accumulations of lymphocytes around necrotic muscle fibres An intracellular thin walled pseudocyst packed with crescent shaped or round parasites about 3–4 microns long and with distinct nuclei rich in chromatin was also seen Its appearance was that of a Toxoplasma pseudocyst No inflammatory reaction was seen around the pseudocyst The groups of lymphocytes in the myocardium probably marked the sites of ruptured pseudocysts

Toxoplasma myocarditis occurs in both an acute and a chronic form The prognosis of the latter is much better than that of the former Cases of myocarditis of unknown origin may be due to Toxoplasma It is however difficult to distinguish between Encephalitozoon Sarcocystis and Toxoplasma

FROM THE DEPARTMENT OF PATHOLOGY I (HEAD GÖSTA HULTQUIST M D) UNIVERSITY OF UPPSALA UPPSALA SWEDEN

GROWTH CURVES, HISTOLOGY AND VIRUS TITRES IN ROUS SARCOMAS

A Comparison Between Progressively Growing and Regressing Tumours

By

BJÖRN STENKVIST[1] and JAN PONTÉN[2]

Received 13 xii 62

Unlike many other tumours Rous sarcomas have a definite tendency to regress spontaneously The percentage of regressing tumours increases with lowering of the virus dose This interesting feature has never been the subject of more extensive studies and the underlying mechanisms are far from understood The aim of this study is to obtain some basic data such as the shape of the growth curve of the tumours, the virus content, and the histology The experiment is in essence a comparison between lethal progressively growing tumours and regressing tumours induced by the same virus dose

MATERIALS AND METHODS

Animals 36 non inbred White Leghorn cockerels 24 days old at the time of RSV inoculation

Virus A 10 per cent saline extract of solid Rous tumours diluted 10^{-3} The RSV strain was originally obtained [illegible]

Inoculation [illegible] inoculated subcutaneously in both wings [illegible]

Growth cu[illegible] [illegible]arly palpated according to a scheme devis[illegible] starting on day 6 Three different diameters of the tumour (length breadth and thickness) were measured with a caliper The tumour volumes were calculated as spheres with the mean of the above three measurements in millimeters taken as the diameter

[illegible] ler Nembutal anes- [illegible] piece was used for [illegible]

[illegible] °C After the ex[illegible] animals whose tumours had regressed and a selection of specimens from the animals which died from progressive tumour growth were thawed and homogenized with 3 ml of phosphate buffered saline (PBS) in a cooled VirTis microcup for 60 seconds After centrifugation at 5000 rpm the supernatant was pipetted off and part of it diluted to 10^{-2} 0 2 ml of

[illegible]

and [illegible]

2 [illegible]

Pa[illegible]

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Growth curves. The inoculation sites were regularly palpated according to a scheme devised by *Bryan* (1956) starting on day 6. Three different diameters of the tumour (length, breadth and thickness) were measured with a caliper. The tumour volumes were calculated as *spheres with the* mean of the above three measurements in millimeters taken as the diameter.

Biopsy. Small pieces of tumours were surgically removed under Nembutal anesthesia. Care was taken to avoid necrotic areas. One half of the piece was used for histology, the other was kept frozen and used *for virus titration.*

Virus titration. The tissue specimens were kept frozen at —65° C. After the experiment was concluded the *specimens fr... m the...*

... centrifugation at ... the supernatant was pipetted off and part of it diluted *to* $10^{-?}$. 0.2 ml of

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[1] This work was supp... and by USPHS resear...

[2] Present Address ... Pa. (USA)

the undiluted extract and the extract diluted to 10 2 was injected into the wing webs of 2 day old test chickens Each chicken receive one inoculation Ten chickens were used for each dilution The chickens used for bioassay were palpated according to *Bryan* (1956), and the latent time until a palpable tumour developed was noted

The individual "titres" were then calculated according to a method described previously (*Ponten* 1956) The present titration differed from the former in that only one inoculation site per chicken was used and that only two different dilutions were injected A correction was made for the weight of the homogenized sample The intersection between the regression line and the dose axis corresponds to the titre value

RESULTS

Growth curves · The response of the 36 experimental chickens with respect to the length of the induction time was quite uniform The mean induction time was 10 5 days At 66/72 inoculation sites a tumour developed 24/36 animals with 45 wing tumours died within 32 days due to rapidly growing tumours in one or both wings 7/36 animals died between days 32 and 84 The tumours in these animals varied in size considerably, but they all eventually regressed

4/36 animals had tumours that regressed and were still alive 327 days after inoculation with no other visible remnants of their tumours than small scars 1/36 animals was consistently negative in both wings

Forty-five progressively growing and lethal tumours from the group that died within 32 days were compared to 7 regressing tumours from four surviving chickens

Already a superficial analysis made two facts clear The host had a decisive influence on the growth behaviour of the tumours This was attested by the fact that no animal carried one progressing and one regressing tumour at the same time The seven regressing tumours were not distributed at random but were all found in four animals The other fact was that in each animal one tumour was always "leading" The tumour that started earliest was at all times larger than the tumour in the other wing that started later

The material has been divided into the following three categories

1) *Progressive tumours* These tumours are defined as growths which never showed a decreasing size and which led to the death of the host within 32 days

The group was divided into two subgroups, the "largest progressive tumours" and the "smallest progressive tumours" In each animal the largest tumour, which always made its appearance earliest, was assigned to the former group, whereas the tumour of the other wing was assigned to the latter group

2) *Regressive tumours* These tumours are defined as growths which after having reached a certain volume decreased until no remnants (except a small scar) remained *These tumours went through a statio*nary phase between days 23 and 27 The animals were still alive 327 days after inoculation

The group was divided into subgroups, "largest regressive tumours" and "smallest regressive tumours" analogously to the progressing tumours

3) Irregular tumours These tumours occurred in animals dying between days 32 and 85 The tumours all eventually regressed after having shown fluctuations in size The cause of death of the animals remains unknown since no autopsies were performed

The tumours of this group were subdivided into "largest irregular tumours" and "smallest irregular tumours" according to the same principle as the two other groups of tumours

The crucial period during which it seemed to be determined whether a tumour would regress or not, was around 23 27 days after virus inoculation Before this period all tumours increased rapidly in size Within and after this period only the progressively growing tumours continued to increase in volume The increase seemed, however, to be slowed down around day 23 27 The regressing tumours went through a stationary phase during the critical period around day 23 27 after which they regressed rapidly

Fig 1 is a comparison between "largest progressive tumours" and "largest regressive tumours" It is seen that during the progressive phase the regressive tumours were on the average somewhat smaller than the progressive tumours The growth rate as expressed by the progression coefficient was essentially similar (0 15 and 0 13) Of interest is the rapidity by which the regression took place The rate of regression (coefficient —0 15) was comparable to the rate of progression in the same group (coefficient 0 13)

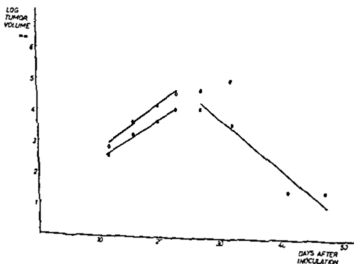


Fig 1

Growth curves of "largest progressive tumours" and "largest regressive tumours" See Text

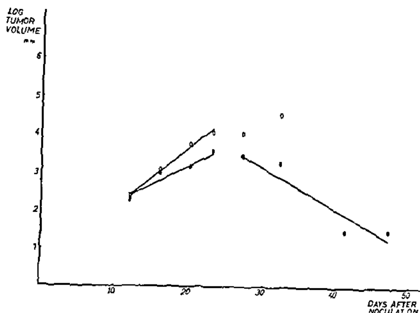


Fig 2

Growth curves of smallest pr gressive tumours and smallest regressive tumours See Text

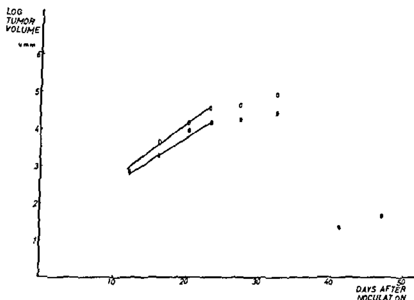


Fig 3

Crowth curves f largest progressive tumours and largest irregular tumo irs See Text

Fig 2 is a comparison between smallest progressive tumours and smallest regressive tumours Again the regressive tumours were somewhat smaller on the average than the progressive tumours at least after day 19 The growth rate was approximately the same (coefficient 0 16 and 0 11) and the rate of regression numerically equal (—0 11) to the rate of progression *in the same group*

Figs 3 and 4 are comparisons between the progressive tumours and

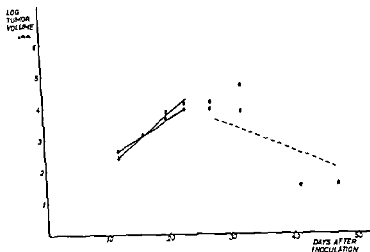


Fig 4
Growth curves of "smallest progressive tumours' and "smallest irregular tumours" See Text

the 'irregular tumours of group 3 During the progressive phase no significant difference was found between the two groups Regression as seen from the wide scatter of the means was more irregular than in the 'regressive' group

Bioassay of virus See Table 1 Eight samples from 6 different progressive tumours were removed between days 13–27 The "titres" varied from 4 1 to 9 3 (mean 7 2)

Seven samples were removed from regressive tumours during their progressive growth phase The biopsies were made on days 13 or 16 The ' titres" varied from 2 4 to 9 0 with a mean of 5 6

Nine samples were analyzed from 7 regressive tumours after their progressive growth terminated, *i e*, after day 23 The biopsies were taken on day 24, 27, 34 The "titres" varied from 0–4 5

Six samples were taken from one of the animals which carried "irregular tumours' all showed high "titres" irrespective of the growth phase of the tumour The values varied from 4 5 and 8 2 with a mean of 6 3

Histology During progressive growth the tumours displayed the well known features of Rous sarcomas (Fig 5) The tumours varied somewhat most tumours were mainly of the spindle-cell type A few, however were dominated by large macrophage like cells rich in cytoplasm There were always areas with necrosis, haemorrhage, and lymphocyte infiltration No difference was noted between tumours that were to regress and those that would continue to grow progressively

TABLE 1

Virus Titres (see text) in Biopsies of Rous Sarcomas in Different Stages of Growth

	Chick number		Titre value on day after inoculation																					
			13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Progressive	9505	R*			9 3												8 7							
		L																						
Tumours	9529	R	6 4														7 2							
		L	9 1						*Progression*															
	9535	R			4 1									5 7										
		L												8 6										
Regressive	9535	R				5 3											4 5							
		L				4 0								2 2										
Tumours	9527	R	9 0																					2 4
		L	6 6						*Progression*					0										1 7
	9530	R				8 1													*Regression*					0
		L				2 4								0										2 6
	9533	R				3 5																		
		L																						0
Irregular	9505	R			6 8									5 5										4 5
		L			6 8									5 9										8 2

* R = right wing web, L = left wing web

Fig 5

Progressively growing Rous sarcoma of typical fibrosarcomatous appearance H & E. × 120



Fig 6

Intermediate stage of regression To the left partly necrotic Rous cells with pycnotic nuclei The middle part of the figure is occupied by a band of *lymphocytes* to the right fibrous tissue H & E. × 120



Fig 7
Advanced stage of regression Fibrous tissue with some vessels of fine calibre and a lymphocytic focus H & E × 120

The histological examination of the regressing tumours failed to show a specific pattern The advanced stage was characterized by fibrous tissue with scattered foci of lymphocytes (Fig 7) In an *intermediate* stage (Fig 6), the tumours were surrounded by a fairly dense band of inflammatory cells, mainly lymphocytes and macrophages Outside this zone, connective tissue was found Even after the tumour was grossly regressing, areas with viable sarcoma cells, some of which were in mitosis, were still seen

DISCUSSION

The objective of the present study was to obtain some basic data about regressing Rous tumours

The most important fact established was the correlation between the state of growth and the content of virus The variation between the individual biopsies was considerable It could, however, be established that *a progressively growing tumour, in general, contained far more* infective virus than a regressing one In fact, in 4 samples from regressing tumours no infective virus at all could be recovered This finding would seem to parallel findings in an ascitic Rous sarcoma where a similar correlation between the state of growth and the titre of cell-associated virus was found (*Ponten* 1956)

The significance of the correlation is questionable It may represent a purely secondary phenomenon The slowly growing or degenerating

cells in a regressing tumour may not be able to produce virus efficiently. On the other hand, the drop in infective virus titre may be the cause of the regression. Recent experiments with sex-marked cells (*Ponten* 1962) have indicated that virus spread and new infection of normal cells may be an important factor for the maintenance of progressive growth in a Rous tumour. If the cells stop producing virus, or if a substantial proportion of the infective particles are neutralized by antibody, regression will eventually result. The finding of little or no virus in the regressing tumours is thus consistent with the hypothesis (*Ponten* in preparation) that the Rous cells are not autonomously growing cells but have a limited life span. A continued release of virus "converting" normal cells into Rous cells may be necessary for sustained progressive growth.

Neither the rate of growth, the histological appearance, nor the virus content of the tumours that grew progressively until the death of the animals or of the tumours that eventually regressed differed significantly until some 25 days after virus inoculation. At that time the tumour seemed to reach a "turning point" after which it either continued to progress or regressed. The factors that determined the fate of the tumour are unknown but seemed to be of host origin. This is concluded from the concentration of all regressing tumours to a few birds and from the observation that no bird carried a progressing and a regressing tumour simultaneously.

SUMMARY

A dilution of Rous sarcoma virus was inoculated into both wings of 36 chickens. The volumes of the ensuing tumours were estimated from repeated measurements of the diameters. Serial biopsies from the tumours were assayed for virus and examined histologically.

The material was divided into two main groups: progressive lethal tumours and regressing tumours. No significant differences with respect to growth rate, content of virus and histology was found between progressively growing tumours that later regressed and those which continued to progress. Tumours in a regressing phase sho...

The findings are compatible with the hypothesis that sustained progressive growth of Rous tumours is normally only posible if normal cells are continously converted into Rous cells by released virus.

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STUDIES ON ODONTOGENIC CYST EPITHELIUM

2 Clinical and Roentgenologic Aspects of Odontogenic Keratocysts

By

J J PINDBORG and JENS HANSEN

Received 23 xii 62

Keratinization of the epithelial lining sometimes occurs in cysts of the jaw Over the last 40 years case reports have sporadically appeared describing such keratinization in follicular, radicular and residual cysts Only recently have larger materials been utilized to study the frequency pattern and general characteristics of odontogenic keratocysts

Philipsen 1956 in a review of available literature suggests the term odontogenic keratocysts for all odontogenic cysts regardless of type showing keratinization of the epithelium This terminology would include keratinized cysts described variously as epidermoid cysts of the jaws (*Brosch* 1938) Jaw cholesteatomas (*Hauer* 1926) (including cysts showing cholesterol splits (*Roussy* 1912 *Rodier* 1920)), multiple cysts of the jaws (*Schlefstein* 1954), and *dysembryoplasies epidermiques des maxillaires* (*Dechaume et al* 1958)

The frequency of odontogenic keratocysts found seems to vary with the type of cyst investigated, and the frequencies reported by various authors therefore reflect the make up of their study material *Langer* (1947) observed a 4 2 per cent keratinization among radicular cysts, Gorlin (1957) 32 per cent among 200 follicular cysts, *Dechaume et al* (1958) 3 3 per cent among 300 cystic lesions of the jaws

The present authors (*Pindborg et al* 1962) reviewing the 1952–1960 records of the Department of Oral Pathology, found 26 (3 3 per cent) keratinized cysts among 791 odontogenic cysts recorded A 17 per cent keratinization was found among residual cysts, 1 per cent among radicular cysts and 7 per cent among follicular cysts Details of the total study material and results of the histological examinations including PAS staining were reported in detail in the first paper

The frequency of these cysts, their roentgenographic similarity to ameloblastomas and other tumors, their liability to recur and the significance of keratinization would seem to warrant a more thorough discussion of their clinical and roentgenographic aspects, which is given here

This study is supported by a grant from the P Carl Petersens Foundation

Colour Plate

A Mallory-stained odontogenic keratocyst with thin epithelium and lumen filled with keratin flakes. × 88.



B Mallory-stained odontogenic keratocyst The parakeratotic layer is red Rete pegs are missing × 360

1

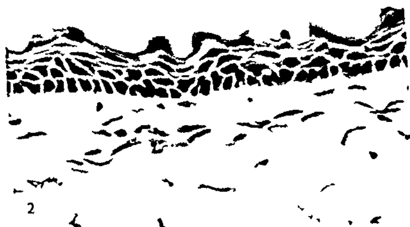


Fig 1

Epithelium from odontogenic keratocyst Note columnar shape of basal cells × 400

Fig 2

Epithelium from odontogenic keratocyst The spinal cell layer is reduced in thickness × 600

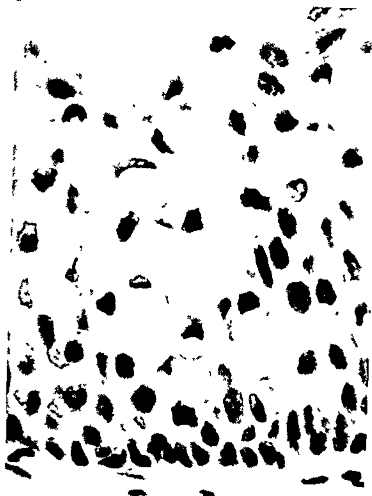


Fig 3
Epithelium from odontogenic keratocyst Note vacuolization of cells in spinal cell layer × 860

Microscopic Characteristics of Odontogenic Keratocysts

The epithelium has a characteristic appearance on histological examination Frequently it is very thin, consisting of 5 or 6 rows of cells, and shows no rete pegs, Colour Plate A The superficial layer of epithelium, *i e* the layer toward the lumen of the cyst, is often wavy The basal cell layer is well defined and is composed of either columnar or cuboidal cells, Fig 1 The stratum spinosum is very scanty (Figs 1 and 2), and sometimes a direct transition of cells from the basal to the superficial layer is observed When a stratum spinosum is present, many of the cells are vacuolated, Fig 3

The keratinization of the epithelium is predominantly of the parakeratotic type It is clearly demonstrated with Mallory's connective tissue stain (Colour Plate B) where the keratinized layers stain bright red against the underlying blue connective tissue cells Despite the

thinness of the epithelium the production of keratin can be extensive. The cyst lumen may be partly occluded by keratin flakes, Colour Plate A.

MATERIAL OF PRESENT INVESTIGATION

Thirty odontogenic keratocysts removed from 27 patients were studied. Most of the cysts were also included in the material reported in the first paper (*Pindborg et al.* 1962).

The patient group comprised an equal number of males (14) and females (13) in an age group ranging from 15–75 years.

FINDINGS

Type and location

Distribution of cysts by type and location is shown in Table 1. Seventeen (57 per cent) of the keratocysts were typed as residual cysts, Fig. 4. A residual cyst is defined as an odontogenic cyst persisting in the jaw after removal of the involved tooth. Seven (23 per cent) were follicular (dentigerous) cysts (Fig. 5), whereas only 1 was diagnosed as a doubtful radicular cyst. Four cysts (13 per cent) were found in relation to vital teeth without any apparent connection with the teeth, Figs. 6 and 7. The location of these cysts in between or apical to normally erupted teeth deems it unlikely that the cysts were derived from primary teeth. One cyst (3 per cent) was classified as a globulo-maxillary cyst.

TABLE 1
Type and Location of 30 Keratocysts in the Jaws

Type	Location		
	Mandible	Maxilla	Total
Residual	14	3	17
Follicular	5	2	7
Radicular (?)		1	1
Globulo-maxillary		1	1
Unknown origin*	4		4
Total	23	7	30

* In relation to vital teeth

Of the 30 cysts, 23 (77 per cent) were located in the mandible, 16 of them in the area of the third molar or the ascending ramus; 7 were located in the incisor, canine and premolar areas of the maxilla. Multiple keratocysts were found in two patients, three cysts in one and two in another.

Roentgenography

The small keratocysts often resembled non-keratinized cysts on roentgenographic examination (Fig. 7), whereas the lager cysts often presented a characteristic picture. Whereas the outer limits of the larger



Fig 4
Schematic drawing of 17 odontogenic keratocysts originated in residual cysts

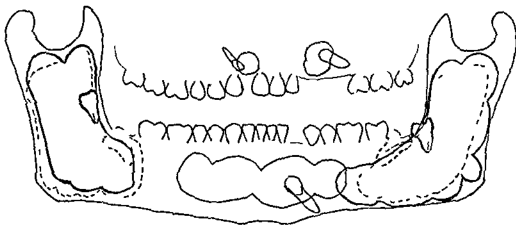


Fig 5
Schematic drawing of 7 odontogenic keratocysts originated in follicular cysts

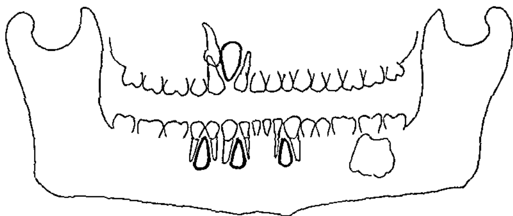


Fig 6
Schematic drawing of 4 keratocysts of unknown origin

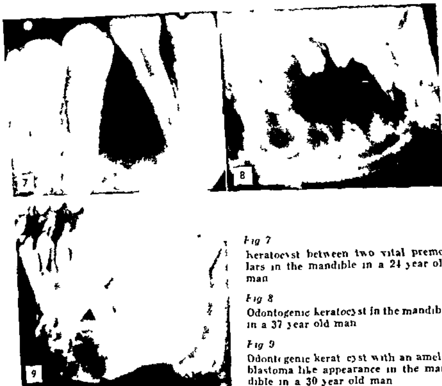


Fig 7
Keratocyst between two vital premolars in the mandible in a 24 year old man

Fig 8
Odontogenic keratocyst in the mandible in a 37 year old man

Fig 9
Odontogenic keratocyst with an ameloblastoma like appearance in the mandible in a 30 year old man

cysts normally were outlined by a narrow, radiopaque structure (Fig 8) some cysts exhibited small, rounded festoons at the periphery, Fig 11 B Moreover the large cysts often appeared multilocular, resembling the structure seen in ameloblastomas and myxomas Fig 9 Strikingly many cysts both in the maxilla and mandible were extremely large, Fig 10 A and B

Macroscopic Findings

During operation the cyst lining was often found of a thinness which made it difficult to obtain enough tissue for histological examination The content of the cyst cavity was often paste like and of a yellowish-white colour This material consisted of desquamated keratin flakes

Recurrence

An attempt was made to evaluate recurrence in the present material Each of the 27 patients was asked to return for re examination 14 patients returned for re examination The remaining 13 includes both those who did not return for re exan [illegible]
rated upon with [illegible]
sented a total o [illegible]
recurred one [illegible] times because of the recurrences the total



Fig 10
A and B Large odontogenic keratocyst in the ramus in a 20-year old woman

amount of operations (original operations + operations for recurrences) was 26 Among these 26 operations 21 were performed more than six months ago 13 of these operations were followed by a recurrence *It means that whether the determination of recurrence rate is* based upon the number of cysts (10 out of 16, 62 5 per cent) or the number of operations (13 out of 21, 61 9 per cent) the result is the same 62 per cent recurrences

In 13 cases the cyst was totally extirpated and in 7 cases a fenestration was performed In one case the method of operation could not be determined There was no correlation between the size or location of the cyst and its tendency to recur, nor was any difference in recurrence observed for the two surgical procedures employed

DISCUSSION

The type and location of keratinized cysts reported in this paper are in general agreement with findings reported by other authors (*Stea* 1960, *Heidsieck* 1953, see also *Philipsen* 1956) and taken as a whole may throw some light on the aetiology of the condition 57 per cent of the keratocysts in our material developed in residual cysts, largely in the mandible Probably most of these cysts have developed from the enamel organ of a molar and have been left when the tooth was ex-



Fig 11

A Lateral radiograph of a recurrent odontogenic keratocyst in the ramus of a 50 year old man
B Laminagram of the same area shown in A Note the festooned margins of the keratocyst

tracted, or in the angle and ramus of the mandible, have grown from distal extensions of the dental lamina, *Heidsieck* 1953, *Offenhauer* 1962 After extraction of the tooth the cysts evidently continue to grow in a quiescent way and often for a long time, in any event they have usually reached a good size when they are diagnosed, cf Figs 4 and 5 13 per cent developed in cysts located in between the roots of vital teeth (Figs 6 and 7), and it might be supposed that these cysts have also developed from remnants of the dental lamina, the so called epithelial pearls of Serres In brief, there seems to be a tendency for keratocysts to develop in cysts with slight or no inflammation of the cyst wall Radicular cysts, developing from inflamed granulomas, rarely become keratocysts in contrast to follicular cysts which often do so An indication of a developmental disturbance may be seen in the occasional occurrence of sebaceous glands in the cyst wall *Dechaume et al* 1958

Shear 1960 suggests that all cysts found in the area of the lower third molar and/or edentulous mandible should be regarded as primordial cysts This postulate may not be justified in view of the large proportion of mandibular residual cysts reported here and elsewhere It is probable that most such cysts actually have developed from remnants of dental structures



Fig 12

Transition of unkeratinized epithelium into parakeratotic epithelium in an odontogenic keratocyst The transition occurs when inflammation disappears × 107

Other peculiarities of odontogenic keratocysts become evident from a study of the literature A number of cases are characterized by being multiple, *Offenhauer* 1962, *Dechaume et al* 1958, *Radden & Janes* 1956, *Catania* 1952, *Schlefstein* 1943, *Caldwell & Thompson* 1955 Both multiple and solitary odontogenic keratocysts may exhibit a familial occurrence, as reported, among others, by *Thoma & Blumenthal* 1946, *Einfeldt* 1961 *Gross* 1953 and *Gorlin & Goltz* 1960 have both described odontogenic keratocysts in patients with multiple nevoid basal-cell epithelioma and bifid ribs with a familial background The material thus far published is still of a case-report nature but indicates that certain individuals may have a hereditary predisposition for a formation of odontogenic keratocysts This predisposition may also include a tendency to develop trichoepitheliomas or basal cell epitheliomas The formation of keratin in cysts as well as in skin tumors is clearly illustrated by *McKelvey et al* 1960 It might well be of value to study more closely the large and accumulating material at hand on patients developing odontogenic keratocysts

The recurrence of odontogenic keratocysts has been emphasized by several investigators (*Catania* 1952, *Heidsieck* 1953, *Einfeldt* 1961, *Offenhauer* 1962) and is striking when compared to the relative rarity of recurring non-keratinized cysts The recurrence rate may be even higher than now supposed as recurring cysts are difficult to detect in

ordinary radiographs Fig 11 A and B illustrates a recurrent odontogenic keratocyst diagnosed by the use of laminography Why keratinized cysts should be more apt to recur than non-keratinized cysts is obscure Serial section of a number of the cysts in this investigation did not show any epithelial proliferation, which could be a possible explanation of a tendency to recur

Some *practical clinical consequences* of the present findings deserve emphasis

1) Roentgenologic examination of odontogenic keratocysts often shows changes that may simulate the presence of a tumor, especially an ameloblastoma The literature shows that an incorrect diagnosis (roentgenologically and histologically) of an ameloblastoma for an odontogenic keratocyst has led to resection of the jaw, *Einfeldt* 1961 A properly evaluated biopsy should be a *conditio sine qua non* in cases of suspected tumor
2) As re examination has revealed a considerable recurrence of odontogenic keratocysts it is very important a) to secure a complete removal of the cyst wall and b) to keep patients under close observation for at least 1 2 years after the operation
3) It is not always possible to make the diagnosis of a keratocyst on clinical and roentgenographic data alone Therefore, all tissue removed from cysts should be subjected to histological examination

SUMMARY

Clinical and roentgenologic findings in 30 odontogenic keratocysts are reported The majority of these cysts are found in residual cysts, next in frequency are follicular cysts A special group comprises cysts in the mandible in relation to erupted vital teeth The roentgenologic appearance is often characterized by festooning margins and a multilocular structure By *re examination of 14* patients and 21 operations it was found that 62 per cent of the odontogenic keratocysts had recurred The practical consequences of these findings are discussed

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DILATED RENAL COLLECTING DUCTS IN A NEWBORN INFANT AS A PRECURSOR TO MEDULLARY SPONGE KIDNEY

Report of a Case

By

ILMARI LINDGREN

Received 19 1 63

The medullary sponge kidney is considered to be a congenital malformation However, only 2 of the 44 patients with medullary sponge kidney were detected before the age 20 in the series of *Ekstrom et al* (1959) There are only two authors who mention the lesion in children under the age of one year (*Lenarduzzi* 1952, *Cabane* 1957) Particularly from the aetiological point of view the registration of the early post natal cases are of importance and in the following a report of a boy aged 4 days with bilateral dilated renal collecting ducts is given These lesions are thought to demonstrate an early phase of medullary sponge kidney

CASE REPORT

The case was a male infant who was the th r l chil [illegible]

13 0 F 1 [illegible]

This work was aided by a grant from "Sigrid Jusélius Stiftelse

[1] The author is indebted to Dr *Maija Miettinen* MD for permitting the case to be reported



Fig 1

Low power view of part of a renal lobe The medulla is composed of dilated collecting ducts × 30

normal and there were no ducts of normal size at the papillary tips The duct became wider towards the papillary tip where their diameter was approximately 0 3 millimetres (Fig 1)

The enlarged ducts contained some cellular debris, which showed a moderate positive PAS-reaction The ducts were never totally filled with the material, which however increased in amount towards the papillary tip The epithelial lining of the ducts consisted of cuboidal cells which showed a tendency to flatten the nearer they were located to the papillary tip (Fig 2) There were no signs of inflammation in the kidney tissue To study the possible mineral deposits in the formalin fixed renal tissue, ultrasoft microradiographs was used at wavelength 8 20 A (*Engström*

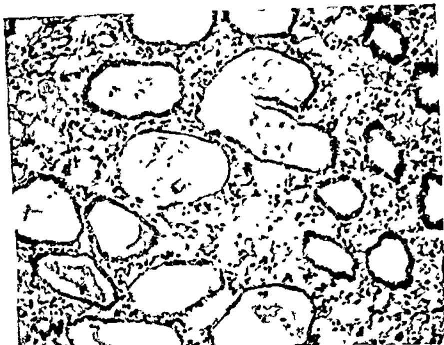


Fig 2

Detail of the center of the medulla showing the dilated ducts with some cellular debris in the lumina × 200

1956) Apart from the presence of small deposits of urates in the cortico-medullary zone no mineral salts were found and the material in the lumina of the ducts had a lower x ray absorption than the soft tissue in general (Fig 3)

In the absence of other changes in the organs examined at autopsy the renal changes were considered the primary cause of death

COMMENT

In their series of 15 histologically examined cases of medullary sponge kidney *Ekstrom et al* (1959) did not find any changes of a type indicating transition to congenital polycystic kidney As the morphological description of early uncomplicated cases of medullary sponge kidney is still lacking no absolute evidence can be obtained if the reported case should have developed to a sponge kidney with large medullary cysts However in this case there were no cysts in the cortex and the lesion clearly differs from the renal dysplasia usually encountered in children (*Ericsson & Ivemark* 1958 *Parkkulainen et al* 1959) this case can be considered as an early phase of medullary sponge kidney The cause of death in this particular case was apparently [illegible]

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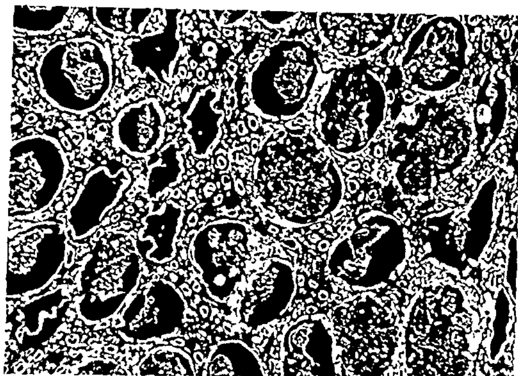


Fig 3

Microradiogram of a 5 micra thick section from the lower part of the medulla Exposed at 8 20 A The material in the lumina has a lower x ray absorption than the epithelial lining of the ducts and the interstitial tissue There are no mineral salt deposits × 180

x-ray-absorbent substances in the ducts, and the absence of inflammatory changes These lesions listed above and lacking in the reported case, should be considered as secondary ones and not typical for the early stage of disease

SUMMARY

The dilated collecting ducts in both kidneys were observed in a male infant aged 4 days at necropsy These lesions were interpreted as an early phase of the medullary sponge kidney described in adults The finding of the lesions in early postnatal life favours the idea that the *medullary sponge kidney is due to a congenital dilatation of the collecting ducts*

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INTRACEREBRAL, POSSIBLY MALIGNANT OSTEOCHONDROFIBROMA IN A CHILD

By

G Flyger H Freidenfeldt and S R Orell

Received 13 xii 62

Primary intracranial tumours with a chondromatous or osteochondromatous structure are very rare (11) The number of such cases described in the literature is at present about 40, principally males between the ages of 18 and 65 Tumours of this type sometimes originate in the base of the skull but most of them are in no way connected with the bones of the cranium They are generally attached to the dura mater or the falx and localized to the convexity (2, 3 4) In certain cases it has not been possible to find any connection between the tumour and the meninges (2, 6) Two cases of intraventricularly situated tumours have also been described (7)

The majority of these tumours are highly diffenrentiated, well encapsulated chondromata with a structure similar to benign chondromata elsewhere in the body (2) Osteochondromata are less common (1, 8, 12) Besides cartilage and bone tissue of varying differentiation certain tumours also contain cellular parts with a fibromatous or meningiomatous structure (5) The tumours are often calcified and are thus visible on plain skull roentgenograms In a few cases, on account of high cellularity, polymorphism and diffuse infiltration into the surrounding tissue the tumours have been regarded as malignant, a fact which has been verified by the clinical course (1, 3 10, 13)

The clinical picture is similar to that of any intracranial tumour correspondingly located though of course it varies according to the speed of growth Growth can be extremely slow in the case of a well differentiated chondroma and much swifter in a tumour which is principally of a fibromatous, cellular nature

D ff [illegible]

of [illegible]

sk [illegible] from embryonal cartilaginous rests in the cranial bones (2) The tumours attached to the meninges are believed by certain authors to originate from embryonal cartilaginous rests in the dura displaced from the base of the skull (5, 8, 9, 12) Other authors claim that they originate from meningeal fibroblasts or



Fig 1 a
Plain skull radiogram showing multiple nodular (↗) and a linear (↗) calcification in the subfrontal region Widening of coronary sutures

multipotent mesenchymal cells in the *dura* or the *arachnoidea* (1, 2, 10) A direct transition from typical meningiomatous tissue to chondromatous tissue has been described, *e g* by *Cushing* (3, 5) Even in the rare cases where there is no visible connection between the tumour and the meninges a connection with the arachnoidea has been assumed (2) The intraventricular tumours are thought to arise from the chorioidal plexus

CASE REPORT

11 ... aged 61) previously healthy and without known
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Otherwise there were no subjective symptoms

Examination at another hospital revealed that the patient's somatic and neurological status was normal apart from ... Plain skull roentgenography revealed considerable ... n the right frontal lobe (Fig 1a) The patien ... tockholm, 1 12 1961



Fig 1b
Ca ot d ang ography early arter al phase Backward upward d slocat on of the an e or cerebral and an er or commun cat ng arter es and of the nfer or part of the per callosal artery The subf ontal vessels are stretched

sub o ta reg on The tumour ontained numerous small calc fications and a t ny l near calc f cat on in its upper posterior border (F g 1) Encephalo graphy was not performed

Operat on was perfo med 18 12 under general anaesthes a w th controll d

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Fig 1 c

Carotid angiography late arterial phase Irregular pathologic vessels Early filling of a large irregular drainage vein

PATHOLOGY

The tumour removed was rounded and measured 5 × 4 × 3½ cm It was solid with a firm almost hard consistence The central part seemed to contain cartilage and bone and was to a great extent calcified while the peripheral parts were softer with a grey white striated cross section The tumour was surrounded by a distinct capsule which however had been somewhat damaged by the operation On the capsule surface there were numerous vessels

Histological examination revealed that a large part of the tumour consisted of highly cellular mesenchymal tissue made up of medium sized spindle shaped cells The nuclei were rather large oval or elongated sometimes vesicular and moderately polymorphous Here and there were a number of mitotic figures The tumour cells were arranged in interwoven bundles but showed no tendency to form whirls or palisading of the nuclei The cellular areas contained very little collagenous intermediary substance Silver impregnation however revealed a fairly manyfold reticulin network There were no psammoma bodies

The central parts of the tumour contained plenty atypical partly calcified osteoid and cellular immature cartilaginous tissue which was also partly calcified The transition from cellular fibromatous tissue to cartilage and osteoid was indistinct In this area the tumour thus was very reminiscent of an osteogenic sarcoma No giant cells were observed The tumour was surrounded by a thin connective tissue capsule and showed no tendency to invade this in the sections examined (Fig 2)

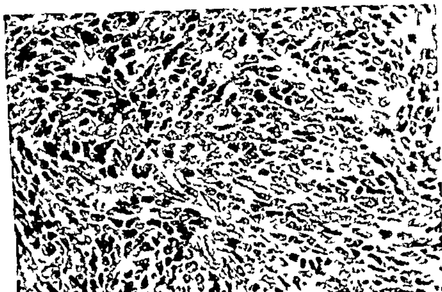


Fig 2a
Highly cellular fibromatous area showing moderate polymorphism
Hematoxylin eosin × 3)0

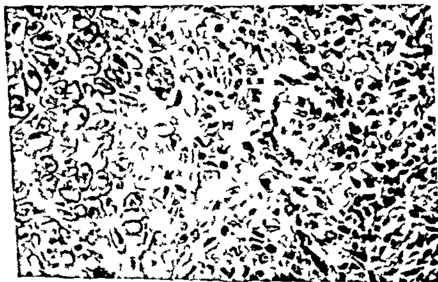


Fig 2b
Showing chondromat us and f bromatous tissue In between atypical osteoid
Hemat xylin eosin × 350

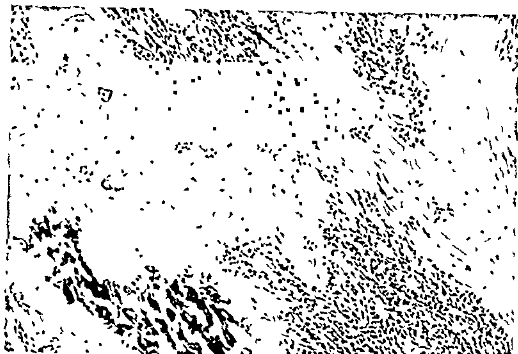


Fig 2c
Cellular chondromatous and osteoid tissue with calcification van Gieson × 100

DISCUSSION

The discovery of a cellular, intracerebrally situated mesenchymal tumour with osteochondromatous components first indicated a metastasis, probably from a primary bone tumour Roentgenographic examination of the entire skeleton and the lungs, however, revealed no pathological changes On account of the roentgenographical picture and the observations made during the operation, the possibility of an osteogenic sarcoma originating from the base of the skull was also ruled out

It was thus a question of a primary intracranial tumour of the same nature as the group of intracranial chondromatous tumours described at the beginning The patient was remarkably young 11 years old So far as we can ascertain there is only one other known case of a tumour of this type in a child *Wolf & Echlin* gave a report of a 9-year-old girl who was operated for an osteochondrosarcoma which was attached to the falx and which infiltrated into both of the frontal lobes The patient died shortly after the operation (13) In our case the tumour was highly cellular and in places fairly rich in mitoses and containing atypical osteoid Histologically it was therefore of a malignant character Judging from the short history, furthermore, it must have grown quickly On the other hand even microscopically the tumour was well defined and easy to remove at the operation Naturally, the period of observation, six months, is still too short to permit a definite prognosis

This case is particularly interesting insomuch as the entire tumour was unquestionably deeply intracerebrally situated and in no way connected either with the falx, the leptomeninges or the base of the skull Nor did histological examination show a structure which could be characterized as meningiomatous Naturally, it is difficult to establish the pathogenesis of the tumour with any certainty To us it seems most likely that it originated from displaced embryonal cell rests of a mesenchymal nature The age of the patient gives a certain support to this theory

SUMMARY

A case is described of an intracerebral tumour with an osteochondro fibromatous structure in an 11-year old boy Histologically the tumour was of a malignant character, highly cellular and containing atypical osteoid, though it was well defined and easy to remove during the operation Six months after the operation the patient was completely free of symptoms Particularly interesting from the pathogenetic point of view was the fact that the tumour was deeply intracerebrally situated without being connected to the dura, the falx or the base of the skull

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ADDENDUM

In june 1963, 17 months after the operation, the patient reported that he was going to school and was perfectly well and free from symptoms

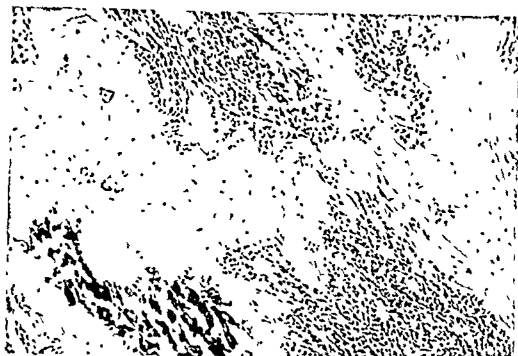


Fig 2c
Cellular chondromatous and osteoid tissue with calcification van Gieson × 100

DISCUSSION

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It was thus *a question of a primary intracranial tumour of the* same nature as the group of intracranial chondromatous tumours described at the beginning The patient was remarkably young 11 years old So far as we can ascertain there is only one other known case of a tumour of this type in a child *Wolf & Echlin* gave a report of a 9-year-old girl who was operated for an osteochondrosarcoma which was attached to the falx and which infiltrated into both of the frontal lobes The patient died shortly after the operation (13) In our case the tumour was highly cellular and in places fairly rich in mitoses and containing atypical osteoid Histologically it was therefore of a malignant character Judging from the short history, furthermore, it must have grown quickly On the other hand even microscopically the tumour was well defined and easy to remove at the operation Naturally, the period of observation, six months, is still too short to permit a definite prognosis

referring to those tumours which are diagnosed in literature as atypical seminoma (*Collins & Schoenenberger* 1962) unusual dysgerminomas or embryonal carcinoma with gonadoblastomatous part (*Santesson & Marrubini* 19;7) These questions areimportant and of great interest but they must be discussed elsewhere

Now I will discuss separately the feminizing form of mixed germ cell tumour (gonocytoma II) and the masculinizing form (gonocytoma III i e gonadoblastoma) In this paper I will try to present both pathological and endocrinological aspects

A FEMINIZING FORM OF MIXED GERM CELL TUMOUR (GONOCYTOMA II)

This tumour is built of germ cells and Sertoli granulosa cells From six previously described cases we can accept the following clinico pathological features of Gonocytoma II (25) The tumour occurs in children and adolescent girls Sex chromatin pattern is positive in accordance with the female character of external genitalia The puberty may either be precocious or normal Menarche may frequently be premature and followed by irregular uterine bleeding No congenital malformations are observed which freluently are found in gonadoblastoma (Gonocytoma III) From the endocrinological viewpoint such tumours are capable of estrogen production resulting in feminization of the patient Most probably Sertoli granulosa cells are responsible for such a hormonal situation

Perhaps the most remarkable case was the one I recently observed (21 25) and which I consider to be typical for feminizing mixed germ cell tumour An 8 year old girl was examined because of symptoms of precocious puberty First vaginal bleeding occurred when she was six years old Bleeding episodes from then or occurred every two to three months Symptoms of premature feminization including womanly stature and fatty tissue topography premature development of breasts and pubic hair growth was noted Gynaecologic examination revealed marked development and pigmentation of the labia minora [illegible]

[illegible] curettings showed proliferative endometrium A year later she was operated on and on the right side an ovarian tumour was found 8 cm in diameter

Histological examination showed lobulated neoplastic tissue composed of cells arranged in round nests separated by partially hyalinized bands of connective tissue (Fig 1) Two types of cells could be recognized 1) germ cells and 2) Sertol gran [illegible]

FROM THE ENDOCRINE DEPARTMENT THE FIRST CLINIC OF OBSTETRICS AND GYNECOLOGY MEDICAL ACADEMY IN WARSAW (HEAD PROFESSOR T. BULSKI)

THE MIXED GERM CELL TUMOURS WITH HORMONAL ACTIVITY

By

JERZY TETER

Received 11.1.63

The mixed germ cell tumours have been recently reported with an increasing frequency (1, 2, 9, 12, 14, 15, 16, 17, 18, 19, 22, 25, 26). The remarkable feature of such tumours is that they are capable of sex hormone production, their hormonal activity may be feminizing or masculinizing.

At the outset I would like to briefly recall my classification of gonocytomas, *proposed in 1960. Since such tumours originate from* gonocytes the term "Gonocytoma" suggested by *Teilum* in 1946, seems to be most appropriate. They were divided into four groups (23).

Gonocytoma I—a homogenous form of tumour formed solely of germ cells (dysgerminoma—seminoma). They are hormonally inactive (20, 21).

Gonocytoma II—a mixed form consisting of two types of cells occurring in the embryonic gonad: germ cells and Sertoli granulosa type cells. These tumours have feminizing properties.

Gonocytoma III—corresponds to Scully's gonodoblastoma and is built of germ cells, Sertoli granulosa cells and interstitial Leydig cells. Calcified concretions in the tumour parenchyma are a conspicuos feature of this class. This group of tumours has masculinizing properties.

Gonocytoma IV—homogenous form of gonocytoma with a marginal *proliferation of androgenic interstitial cells* (analogous to the theca-lutein reaction seen in the ovarian stroma surrounding metastatic rests of Krukenberg's tumours.

The possibility of pre-operative diagnosis in such tumours as well as the problem of differentiation between feminizing and masculinizing forms are the subject of this lecture.

I would also like to note, that certain rare neoplasms occur which most probably belong to mixed germ cells tumours but whose very complex cyto-architecture makes the exact diagnosis impossible. I am

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Presented by *invitation, before the Joint Meeting of Pathologist and* Endocrinologists Copenhagen December 1962.

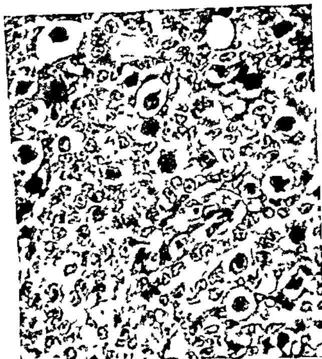


Fig 1 b

Feminizing form of mixed germ cell tumour (Gonocytoma II)
Area of a confusing a admixture of germ cells and sex cord cells in malignant fashion

aged 15 operated on in our Clinic in 1938 The other one observed by Dr Hughesdon from London of a 16 year old girl with symptoms of metrorrhagia Both were described in my previous report (25) together with three cases from pre war literature It is noteworthy that all cases of gonocytoma II collected from literature occurred in children and adolescent girls (23) Uniformity of clinical and pathological features of the described type of tumours enables their classification as a definite clinico pathological entity feminizing mixed germ cell tumour (Gonocytoma II)

A MASCULINIZING FORM OF MIXED GERM CELL TUMOUR (GONOCYTOMA III)

This kind of tumours was originally described by *Scully* in 1953 and given the name of gonadoblastoma since it was found to recapitulate the embryonic development of the gonad better than does the pure gonocytoma

The neoplasm is composed of germ cells Sertoli granulosa and interstitial Leydig cells Its conspicuous feature is the presence of calcified concretions in the parenchyma The tumour occurs in patients with

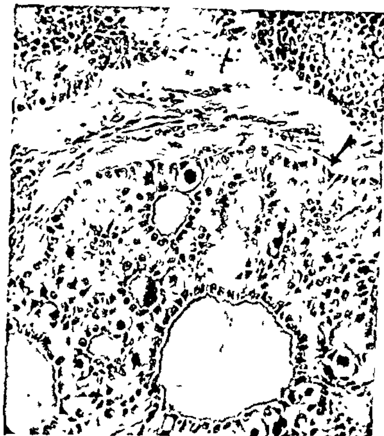


Fig 1 a

Feminizing form of mixed germ cell tumour (Gonocytoma II)
Single germ-cells scattered inside the nest are most closely related to the Sertoli than to the granulosa (arrow) type In the other region sex-cord cells differentiate toward granulosa cells and form a microfollicular pattern Connective tissue bands separate the neoplastic nests and contain only a few spindle shaped cells

losa cells and in some places were encircled by them Small areas of the tumour were characterized by a confusing mixture of germ-cells and sex-cord cells in malignant fashion (Fig 1 B) Connective tissue which separated neoplastic nests contained only few spindle-shaped cells The stroma did not contain any Leydig cells Unlike cases of embryonal tumours of testicular type (Scully's gonadoblastoma or Melicow's gonadoma) calcified concretions and clumps of Leydig cells are never found here

The histological investigation showed that in certain areas the sex-cord cells were more closely related to the Sertoli than to the granulosa type of cells (Fig 1 A) It is known that in human beings Sertoli-cell tumours of both testis and ovary are feminizing (*Teilum* 1958, *Morris & Scully* 1958) On the other hand microscopic studies also disclosed that sex-cord cells differentiated in some regions toward granulosa cells and formed a microfollicular pattern (Fig 1 A) resembling folliculoma Such tumours usually produce also feminizing effects

Two similar cases of Gonocytoma II may be quoted One is of a girl



Fig 2c

Masculinizing form of mixed germ cell tumour (Gonocytoma III Gonadoblastoma) Microscopic picture of a section from the tumour of the right gonad Typical foci of calcification and small nest of neoplastic cells

somato sexual disturbances (eunuchoidal features or signs of Turner s syndrome) reared as women and having female type of external genitalia but with symptoms of masculinization In all reported cases of gonadoblastoma negative (male) sex chromatin pattern was found (see Table 1) From the endocrinological viewpoint such tumours are capable of sex hormone production mainly of androgens

Recently we reported a new case of gonadoblastoma (26) which I consider most illustrative for the masculinizing group of mixed germ cell tumour A patient aged 21 reared as a woman was examined because of primary amenorrhea retardation of sexual development and virilism Two uterine bleeding episodes occurred when she was 20 She was a manly statured female with eunuchoidal body proportions Acne seborrhea slight temporal baldness were present as well as signs of facial and body hirsutism The breasts were of pubertal type The clitoris was hypertrophied the vagina well formed the uterus was small



Fig 2a

Masculinizing form of mixed germ cell tumour (Gonocytoma III Gonadoblastoma) Hysterosalpingogram The gonadal tumour contained faint floccular foci of calcification Small uterine cavity and patent fallopian tubes

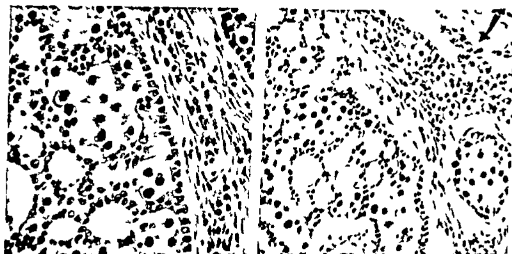


Fig 2b

Masculinizing form of mixed germ cell tumour (Gonocytoma III Gonadoblastoma) Microscopic findings in a section from left dysgenetic gonad showing typical gonadoblastoma pattern Note the two large nests composed of Sertoli granulosa cells The former are the small cells with oval carrot shaped nuclei arranged in a single row along the periphery of the nest which form a folliculoid pattern or encircle the germ cells The latter have *large nuclei and clear cytoplasm Between the nests* small clumps of immature Leydig cells are seen

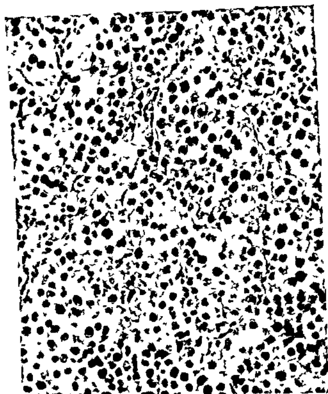


Fig 2 e
Masculinizing form of mixed germ cell tumour (Gonocytoma III Gonadoblastoma)
A large nest of germ cells similar to pure dysgerminoma"

on the right, behind the uterus It contained faint floccular foci of calcifications (Fig 2 A) This finding with all the above evidence, enabled us to diagnose this tumour, before the operation—as a gonadoblastoma

Laparotomy disclosed an infantile uterus and fallopian tubes On the left a typical dysgenetic, whitish streak in the place normally occupied by the ovary, and on the right a gonadal tumour were found The tumour measured 5 by 3 by 2 5 cm was covered by a yellowish gray, smooth glistening capsule and contained chondroid like nodules On the fourth postoperative day uterine bleeding occurred The histological examinations revealed that the cortical part of the left dysgenetic gonad built of scanty connective tissue, resembling ovarian stroma In the medullary part a small nodule was found, and recognized as incipient gonadoblastoma [illegible]
cells scattere [illegible]
in three typ [illegible]
around germinal cells, 2) in peripheral single file along the base of the



Fig 2 d
Masculinizing form of mixed germ cell tumour (Gonocytoma III Gonadoblastoma) Calcified concretions and groups of Leydig cells

and infantile Sex chromatin pattern was negative (male) Urinary 17-ketosteroid output was within normal limits (6 7–10 1 mg/24 hrs) Slight estrogen activity was found both in the urine (23 mcg by *Jayle's* chemical method) and in vaginal smears Gonadtropin level was increased to 66 and 71 m U (*Kleinfelter-Dekanski* method) Human chorionic gonadotropin stimulation with simultaneous inhibition or adrenal androgenic activity by prednisone, provoked a significant increase in estrogen excretion from 23 to 47 72 mcg The 17-KS output was only slightly affected (6 9 8 8 mg/24 hrs) This response resembled the result of HCG stimulation observed in testicular dysgenesis (3, 5, 8, 9, 10, 11) The possibility of the presence in this case of testicular dysgenesis was supported by the evidence of negative sex chromatin pattern and symptoms of intersexuality with some masculinizing features

Radiological examinations were decisive for the diagnosis Hysterosalpingogram demonstrated a small uterine cavity with patent fallopian tubes (Fig 2 A)

The most striking and surprising disclosure was a tumour situated

References	Scully's cases 1953		Ber 1948	Przybora 1960	Siebenmann 1961	Author's cases 1960	Author's cases 1962
	No 1	No 2				No 1	No 2
No of case	1	2	3	4	5	6	7
Age	8	19	25	23	13 5	19	21
Habitus	girl	andr "girls	eunuchoidal and androidal	eunuchoidal and androidal	androidal	Turner and 'boyish'	eunuchoidal and androidal
Uterus and Fallopian tubes	?	+	+	+	+	+	+
Gross pathology							
a) right gonad	tumour	tumour	rudiment	tumour	testis-like tumour	tumour	tumour
b) left gonad	? +	—	tumour	dysgen streak	rudiment	dysgen streak	dysgen streak
Microscopic findings							
Characteristic nest of germ cells and sex cord cells	+	+	+	+	+	+	+
Leydig cells							
a) clumps	+	+	+	++	+	++	+
b) adenoma-like formation				+		+	
Calcified concretions	+	+	+	++	+	+	++
Areas of 'pure gonocytoma	+	+	+	+	—	+	+

All patients had female legal sex female type of genitalia and negative sex chromatin pattern together with androgenic symptoms (hypertrophied clitoris and hirsutism).

* Classified as dysgerminoma in original report (*Ber* 1948) The sex chromatin in this case was established by examining various nuclei in the slides of the operative specimen

§ The author is greatly indebted to Dr *Scully* (Boston USA), Prof *Ber* (Tel-Aviv, Israel), Dr *Siebenmann* (Zürich, Switzerland) and Dr *Przybora* (Poznan, Poland)—for sending slides and clinical data (reprints) from their cases of gonadoblastoma and their permission to use them in my researches

pseudotubules, 3) in folliculoid pattern (Fig 2 B) Foci of Leydig cells were clustered between the cellular nests described above

The tumour of the right gonad presented *a more typical structure of* gonocytoma III (gonadoblastoma, Fig 2 C) In many areas microscopic examination showed in the hyalinized stroma small groups of Leydig cells and *calcified concretions in form of irregular masses or "psammoma-like"* bodies (Fig 2 D) In other parts the neoplastic nests were composed solely of germ cells in an arrangement typical for "pure dysgerminoma" *(gonocytoma I, Fig 2 E) In still other portions of the tu*mour Sertoli-granulosa cells dominated

The following symptoms showed that the tumour was capable of *sex-hormone production Four weeks after the operation urinary estro*gen output decreased from 23 mcg to about 5 mcg/24 hrs The urinary 17-KS were lowered from 10 1 to 5 6 mg/24 hrs The patient complained *of sweats and hot flushes These symptoms disappeared after replace*ment therapy was instituted and cyclic uterine bleeding ensued

I collected as many clinical data as possible together with microscopic *slides from seven gonadoblastoma cases, which have been reported in* recent years Table 1 presents some clinical, hormonal and pathological features of these cases Endocrine signs from my two cases have been *collected in detail in Table 2*

The material presented proves, that there exists a uniformity of clinical and pathological features of this type of tumours which enables *their classification as a definite clinico-pathological entity* masculinizing mixed germ cell tumour (Gonocytoma III) Even more, it allows detection and a preoperative of gonadoblastoma may be listed as follows

1) Abnormal somato-sexual development in a patient reared as a woman with primary *amenorrhea and some signs of masculiniza*tion

2) Negative (male) sex chromatin pattern, contrasting with female type of genitalia

3) High gonadotropin urinary titre despite clinical and biochemical *signs of sex-hormone* activity (analogous with cases of testicular feminization syndrome)

4) A significant increase in estrogen excretion after HCG stimulation (normally observed *in men or in case of testicular dysgenesis* (3, 4, 8, 10, 11))

5) Radiological evidence of a gonadal tumour containing floccular foci of calcification The usefulness of such radiological examination in detection of gonadal neoplasm in patient with congenital somato-sexual ambiguities was first shown by *Melicow & Uson* (1959)

The degree of masculinization seems to be dependent on the relative number of Leydig cells (Fig 2 B and 2 D) The masculinization features (enlarged clitoris and scrotal appearance of the labia) were most marked in my first case of gonadoblastoma (Table 2) The characteristic histological picture was in full accord with this clinical features In many sections foci of Leydig cells were seen in hyalinized stroma The most marked finding, in my opinion were circumscribed agglomerations of Leydig cells in the form of small adenomas, which appeared to be hyperplastic

On the other hand, however, oestrogenic symptoms were also observed (Table 1 and 2) which probably were dependent of the Sertoli-granulosa cell component In my two cases the characteristic withdrawal bleeding occurring after removal of the tumour, proved, that the gonadoblastomas are also capable of estrogenic production It should be noted however, that in these two cases ostrogenic symptoms were not as marked as the androgenic features

A separate question is that of high gonadotropin urinary values in cases of gonadoblastoma The increased excretion of gonadotropins in cases of testicular tumour, as reported by *Hamburger*, was probably due to a reduction of the total amount of normal testicular tissue In cases of gonadoblastoma dysgenetic, defective gonads and tumours tissue are unable to produce enough sex hormones to supress the release of gonadotropin by the pituitary

SPECIAL PATHOLOGICAL ASPECTS

Testicular Dysgenesis as a Background for the Development of masculinizing Form of a Mixed Germ Cell Tumour

Several histological features may indicate that gonadoblastoma arises in and from dysgenetic or rudimentary testes It will simplify matters if these phenomena are collected in three groups

1) *Scully's* two first gonadoblastomas presented the unusual feature of disseminated milliary *calcification* which in his experience does not characterize the pure dysgerminoma Similar formations in all later reported gonadoblastomas are found as *psammoma* like bodies or calcified fleks or irregular masses (Fig 2 C, 2 D, 3)

In the light of present investigations it can be [illegible]

[illegible] *Stange* 1960, *Teter & Tarlowski* 1960, case No 2 *Dominguez & Greenblatt* 1962) [illegible]

[illegible] *Zillinger & Westman*

ENDOCRINOLOGIC ASPECTS OF GONADOBLASTOMA CELLULAR SOURCE OF SEX-HORMONE PRODUCTION

In clinical pictures of gonadoblastoma androgenic symptoms are always observed (see Table 1) and seem to be the dominating factor The androgenic activity of such tumours is due to the interstitial Leydig cells component In all slides of the seven studied cases I was able to observe interstitial Leydig-like cells, varying in number, appearance, size and arrangement. They actually contain considerable lipochrome pigment in their cytoplasm, which is characteristic of leydig cells I agree with *Scully*[1] that they are most probably Leydig and not theca cells. I should however add, that I never saw any typical crystalloids of Reinke in these cells, but this is almost the general rule in testicular dysgenesis

TABLE 2

Endocrine Symptoms and Their Morphological Basis in two Cases of Gonadoblastoma (Gonocytoma III) Observed by the Author

Characteristics	Case I previously published (1960)	Case II (1962)
I *Symptoms of endogenous oestrogen production*		
1 Breast development	underdeveloped pubertal type	pubertal type
2 Vaginal development Ability to have sexual intercourse	rather poor	good
3 Spontaneous uterine bleeding	none	Two episodes of bleeding at 20 years of age
4 Withdrawal bleeding after gonadectomy (removal of the gonadal tumour)	on 8th post-operative day	On 4th post-operative day
5 "Climacteric" symptoms following operation	present	present
II *Symptoms of endogenous androgen production*		
1 Enlarged clitoris	prominent	present
2 Appearance of the labia maiora	scrotal	female
3 Tendency to hirsutism	*present*	present
4 Skin	pale and oily	pale and oily
III *Characteristics of cells capable of hormone production*		
1 Sertoli-granulosa type cells	present	present in abundance
2 Interstitial Leydig cells	dominating in large groups	singular, scattered
a) appearance, size	medium sized polygonal	small, polygonal
b) special arrangement	areas of adenoma-like formations	small clumps

1 *Scully* personal communication

SUMMARY AND CONCLUSIONS

1) It is claimed that there exist two separate and definite forms of mixed germ cell tumours feminizing (gonocytoma II) and masculinizing (gonocytoma III—corresponding to Scullys gonadoblastoma) They differ in the clinical, endocrinological and pathological aspects

2) Feminizing form of mixed germ cell tumours occurs in children or adolescent girls, with a normal somato sexual development, but often with symptoms of precocious puberty or acyclic uterine bleeding The neoplasm is composed of germ cells (hormonally inactive) and Sertoli granulosa cells, which are the cellular source of estrogens

3) Masculinizing form of mixed germ cell tumours occurs in patients with somato sexual disturbances (eunuchoidal features or signs of Turner s syndrome) having female type of external genitalia but also symptoms of masculinization, and with negative sex chromatin pattern The neoplasm is composed of three kinds of cells germ cells, Sertoli-granulosa cells (cellular source of estrogens) and interstitial Leydig cells (cellular source of androgenic effects could be observed Androgenic effects were found to be dominant It must be noted, that some histological features may indicate that gonadoblastoma (gonocytoma III) arises in and from dysgenetic or rudimentary testes

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2) In my first case of gonadoblastoma the tumour was on the right side, and on the left a dysgenetic streak was found, in which the medullary region contained rudimentary seminiferous tubules On the other hand microscopic investigations of the tumour disclosed in some areas nests of Sertoli-type cells and germ-cells in an arrangement resembling seminiferous tubules

3) Most interesting aspects are presented in Siebenmann's case of a *male hermaphrodite with a gonadoblastoma* The *tumour* was discovered at the periphery of definite testicular tissue There was no sharp dividing line between the typical testicular tissue and the gonadoblastoma To the contrary one could observe gradual transitions from seminiferous tubules to gonadoblastoma nests

Lastly I would like to mention the results of HCG stimulation test The increased excretion of estrogens after HCG stimulation suggests the presence of testicular tissue in the gonadal tumour, particularly in a patient with negative sex chromatin pattern According to *Maddock & Nelson* (1952), *Jayle et al* (1958), *Diczfalusy* (1958), *Dux* (1956) such an estrogen response is specific for testicular tissue



Fig 3



Fig 4

Fig 3 Psammoma like bodies and the *calcified concretions* surrounded by hyalinized connective tissue with small clumps of Leydig cell (Gonocytoma III own case No 1)

Fig 4 Psammoma like calcification in testicular dysgenesis (case of male hermaphrodite B H aged 21 specimen No 20748)

FROM THE VETERINARY BACTERIOLOGICAL LABORATORY VASTERAS AND THE STATE BACTERIOLOGICAL LABORATORY STOCKHOLM SWEDEN

THE ANTISTAPHYLOLYSIN TITRE IN CATTLE AFTER SUBCUTANEOUS AND INTRAMUSCULAR TREATMENT, RESPECTIVELY, WITH A COMBINED STAPHYLOCOCCAL VACCINE

By

H THORNE CAROLA NEUMULLER and P O NILSSON

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Staphylococcal mastitis is a comparatively common disease in cows and in many dairy herds it is the predominating infection (13 14) Since this infection offers therapeutic problems hopes have been raised of active immunization During recent years several reports on immunization trials have appeared (1 2 4 5 6 7 8 9 10 12) In these experiments toxoids alone or toxoids together with adjuvants have been used Treatment with different vaccines has stimulated the production of immune bodies to varying extents (8 9) Individual differences in the treated animals have also been noted In young animals for instance the rise of the antistaphylolysin titre has proved to be less marked than in older ones (12)

The purpose of the investigation presented here was to study possible differences in the rise of the antistaphylolysin titre on the one hand after subcutaneous vaccination on the other hand after intramuscular vaccination

MATERIAL AND METHODS

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RESULTS

The antistaphylolysin titre before and two weeks after treatment is iven in Table 1 The same result expressed by cumulative values is lustrated in Fig 1

From the table and the figure, it is seen that the serum titre in the 6 animals varied initially betwen < 0 36 and 4 0 I U/ml of blood seum In most of the animals the serum titre showed a considerable rise wo weeks after treatment The rise was higher, as a rule, in the 23 nimals that had been treated intramuscularly than in the 43 that had een vaccinated subcutaneously Statistically, the difference was highly significant ($P = < 0\ 001$)

DISCUSSION

In immunization experiments with staphylococcal toxoid in general a varying rise of the antistaphylolysin titre has been noted The variations have been attributed to differences in the vaccines used and to differences in the treated animals as producers of immune bodies In the investigation presented here two comparable groups of animals were treated with the same vaccine, one group subcutaneously and the other intramuscularly with the purpose to detect possible differences in the immune response to the two ways of application In both groups a rise of the antistaphylolysin titre in blood serum could be noted The rise was significantly higher in the group treated intramuscularly

The possibility that the result might have been influenced by a nondiscovered staphylococcal infection in one group can be excluded The animals of each group were distributed into several different herds Moreover, a large number of control animals in each herd did not show any rise of the serum titre

Besides, the injection of vaccine was performed in such a manner and with such care that leakage after treatment must be excluded

Consequently, a definite relationship existed between way of application and rise of the antistaphylolysin titre in blood serum The result inspires to continue studying immunity response for longer periods of time after subcutaneous and intramuscular treatment, respectively

Even if the antistaphylolysin titre hardly can be interpreted as an actual indicator of an organism's state of immunity, the aim of treatment with toxoids is generally, however, to obtain the greatest possible *rise of the serum titre On the* basis of the present results, intramuscular application may be recommended as preferable to subcutaneous in toxoid treatment of cattle

SUMMARY

Two groups of cows were treated with a combined staphylococcal vaccine In one group the vaccine was injected subcutaneously and in

TABLE 1

Antistaphylolysin Titre in 43 Cows Vaccinated Subcutaneously (Sc) and in 23 Cows Vaccinated Intramuscularly (Im) before and two Weeks after Treatment, Respectively

Antistaphylolysin titre		<0 36	0 36	0 5	0 7	1 0	1 4	2 0	2 8	4 0	5 6	8 0	11	16	22	32	44	64	88	128
Sc	Before	7	2	9	3	9	1	7	5	–	–	–	–	–	–	–	–	–	–	–
	After	1	–	1	–	5	2	11	2	5	2	5	3	5	–	1	–	–	–	–
Im	Before	3	1	7	–	8	1	–	1	2	–	–	–	–	–	–	–	–	–	–
	After	–	–	–	–	–	–	–	1	3	2	4	1	5	2	2	2	–	–	1

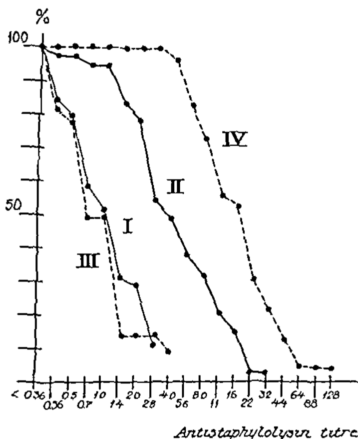


Fig 1

Abscissa Antistaphylolysin titre (I U.)
Ordinate o/o animals, each dot gives the percentage of animals in the group that has a corresponding or higher antistaphylolysin titre
Curve I = Antistaphylolysin titre in 43 animals
Curve II = Antistaphylolysin titre in the same 43 animals two weeks after subcutaneous vaccination
Curve III = Antistaphylolysin titre in 23 animals
Curve IV = Antistaphylolysin titre in the same 23 animals two weeks after intramuscular vaccination

RESULTS

The antistaphylolysin titre before and two weeks after treatment is given in Table 1 The same result expressed by cumulative values is illustrated in Fig 1

From the table and the figure, it is seen that the serum titre in the 66 animals varied initially betwen < 0 36 and 4 0 1 U/ml of blood serum In most of the animals the serum titre showed a considerable rise two weeks after treatment The rise was higher, as a rule, in the 23 animals that had been treated intramuscularly than in the 43 that had been vaccinated subcutaneously Statistically, the difference was highly significant (P = < 0 001)

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SUMMARY

Two groups of cows were treated with a combined staphylococcal vaccine In one group the vaccine was injected subcutaneously and in

the other intramuscularly Two weeks after treatment the rise of the antistaphylolysin titre in blood serum was higher in animals treated intramuscularly The difference was statistically significant

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FROM THE NATIONAL BACTERIOLOGICAL LABORATORY STOCKHOLM SWEDEN
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TECHNIQUE OF VOGES-PROSKAUER TEST

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The importance of a uniform and correct technique for the IMViC test was pointed out in an earlier paper (*Ljutov* 1959) and a method for testing the indole reaction was suggested The methyl red test was dealt with in a later study (*Ljutov* 1961) The present paper will be devoted to the Voges Proskauer (VP) reaction and is one of a series of studies of the technique of the IMViC test

Voges & Proskauer observed in 1898 that the addition of KOH to certain bacterial cultures produced an opalescent reddening especially of the upper part of the culture after it had been standing for 24 hours at room temperature In 1901 *Grimbert* and in 1904 *Desmots* (according to *Harden & Walpole* 1906) discovered that the red colour in the VP reaction is caused by acetylmethylcarbinol (acetoin) which is formed by Aerobacter aerogenes among other bacteria In the presence of caustic alkali and air the acetylmethylcarbinol (CH_3 CH (OH) CO CH_3) is oxidized to diacetyl which in turn reacts with other substances possibly arginine to produce the red colour (*Barritt* 1936)

To increase the sensitivity of the VP reaction O Meara recommended in 1931 the addition of creatine to cultures for testing of the VP reaction In 1936 Barritt proposed the addition of α naphthol which had a better effect than creatine

According to Barritt the VP test should be performed by adding 0 6 ml of a 5 per cent solution of α naphthol in absolute ethyl alcohol and 0 2 ml of a 40 per cent solution of KOH to 1 ml of culture which is thereafter agitated A positive reaction appears as a pink colour after 2 5 minutes The colour attains its maximum intensity within an hour and becomes raspberry red or ruby red in strongly positive and aniline or light red in faintly positive reactions Negative reactions remain colourless or have a faint copper colour

In the studies of the VP reaction described in the sequel a more thorough consideration will be given to Barritt s method as being the most sensitive

METHOD AND MATERIAL

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the medium a yellowish hue which interfered with the reaction The sterility was thereafter checked by incubation of the medium at 35–37° C for 24 hours

In view of the observation by *E Taylor* (1958) that dextrose phosphate substrate can in itself produce positive VP reactions by Barritt's method the present author tested 104 batches of sterile medium in this respect All batches produced a negative reaction by Barritt's method

The following five kinds of *peptone* were used for testing the dextrose phosphate medium· 1 Wilson & Co, Chicago, 2 Witte, Rostock; 3 Orthana Copenhagen, 4 Difco Proteose B 122, Detroit, 5 "indolefree" peptone, Schmitt-Jourdan, Boulogne sur-Seine

Lactose peptone broth with indicator (Liebig's extract 3 g, lactose 10 g peptone Orthana 10 g, NaCl 3 g, 1 per cent bromcresol purple in alcohol 1 ml, and aq dest *1000 ml) was used* for determining the production of acid and gas by coliform bacteria

Two different methods were employed for *inoculation of the cultures* In one the bacteria were grown first in broth, after which tubes containing dextrose phosphate were inoculated with 0 2 ml of this broth culture diluted to 10 1 (*method A*) In the other the material was transferred with a loop from agar slants or Endo agar plates to the dextrose phosphate tubes The loop was dipped in the medium once (*method B*)

5 per cent *a naphthol solution* was used in 95 per cent ethyl alcohol since it produced stronger reactions than any kind of absolute alcohol solution Numerous experiments showed that *the first solution does not lose its strength after storage* for one month at room temperature The flasks were stoppered with screw on bakelite caps The α-naphthol (melting point 95 97° C) derived from Hopkin & Williams Ltd, England

The material studied consisted of 1532 coliform strains from well water and 153 from faeces Gram-negative non-sporing rods which fermented lactose under development of acid and gas were regarded as coliform strains

EXPERIMENTAL

Experimental studies have shown that the kind of peptone used in the dextrose phosphate medium, the temperature and time of incubation, and the testing technique, all play an important part The experiments were often made with several simultaneous goals in view To simplify the presentation of the results, each question will be dealt with separately below

Different kinds of peptone The original recommendation, by *Clark & Lubs* (1915), was that Witte peptone alone should be used for the MR test The same peptone was used for the VP test Later other kinds of peptone were suggested For the choice of the best kind of peptone, five peptones in dextrose phosphate medium were compared in the cultivation of 258 coliform strains inoculated by method A The strains were grown at nine incubation temperatures between 27° and 45° C The results are shown in Table 1 Differences between the *various peptones*, though slight, were observed even at 27° C Peptone 1 gave the largest number of positive VP reactions in strains both from well water and from faeces Peptone 5 gave almost as many positive reactions, peptones 3 and 4 a smaller, and peptone 2 the smallest number The differences between the peptones were most manifest at higher incubation temperatures At 45° C incubation of the faeces strains, acetoin was best formed in dextrose phosphate with peptones 1 and 5, but not at all with peptone 2 The best types of peptone were considered to be 1 and 5

TABLE 1

Different Kinds of Peptone Number of Strains Showing Positive VP Reactions

	Kinds of peptone	Incubation temperatures								
		27°	30°	33°	35°	37°	39°	41°	43°	45°
218 strains from water	1	198	196	194	189	177	7			
	2	192	184	180	162	123	9			
	3	196	192	188	169	146	22			
	4	190	189	186	168	138	24			
	5	196	196	193	185	172	22			
40 strains from faeces	1	39	39	38	36	35	34	20	19	16
	2	37	36	33	24	20	10	1		
	3	37	36	37	36	35	20	12	10	6
	4	38	36	36	34	29	21	15	14	8
	5	38	37	37	36	34	28	17	17	14

Negative reactions with faint copper colouration occurred in certain strains cultivated in dextrose phosphate, usually with peptone 1 The higher the incubation temperature, the more vivid was the copper colour of the strains

Incubation temperature As seen from Table 1, incubation at 27° C gave a larger number of positive VP reactions than at 30° C and above, irrespective of the type of peptone used in the dextrose phosphate medium

Tests by method B were also made on 126 coliform strains isolated from water The results confirmed that culture at 27° C gives the largest number of positive VP reactions

Positive reactions seldom ceased suddenly at the transition from lower to higher incubation temperatures As a rule very weak light pink reactions were observed between positive and negative reactions (*e g* 33° C + + +, 35° C ±, 37° C —) The VP reaction was usually found to be negative when no macroscopically visible growth occurred in the culture, but also at a high incubation temperature, despite the sometimes strong growth in the culture

Coliform bacteria from faeces produced varying patterns of positive VP reactions Some strains showed positive reactions up to 45° C, others formed acetoin at 43°, 41° or lower temperatures Certain faeces strains produced acetoin only at 27° C Coliform bacteria from water showed positive reactions only up to 39° C or below

Incubation period To ascertain the most appropriate period of incubation for the VP test, 193 coliform strains from water were examined by method A Each strain was inoculated into four tubes containing dextrose phosphate which were then incubated at 27° C After 24, 48, 72 and 96 hours of incubation, respectively, one tube was removed from the incubator and examined for VP reaction The results are shown [illegible]

TABLE 2

Incubation Period for VP Test

Days of incubation	1	2	3	4	Number of strains
	+	+	+	+	141
	—	+	+	+	9
	—	—	+	+	5
	+	+	+	—	2
	—	—	—	—	36
Total positive reactions	143	153	157	155	
Total negative reactions	50	41	36	38	
	193	193	193	193	193

Incubation for so long a time as four days is inadvisable owing to the risk of splitting of acetoin (*Paine* 1927, *Williams et al* 1928, and *Tittsler* 1938) The choice remains between two and three days of incubation To settle this point, 1066 coliform strains from well water were inoculated by method B after membrane filtering and isolation on Endo agar plates The highest and lowest incubation temperatures for acid and gas formation, were determined and the IMViC test was performed for each strain Two tubes were then inoculated and incubated at 27° C One tube was examined for acetoin formation after two and the other after three days All strains except seven showed similar results after two and three days of incubation These seven strains (0 6 per cent) were retested by method A four were found to correspond to the results of two days and three strains to three days of incubation at the first examination From the practical point of view, therefore, there was no difference between two and three days of incubation

The conclusion was that two or three days of incubation at 27° C is the optimum period for the VP test

Technique of VP test In the experiments described below, each strain was inoculated by method A into five dextrose phosphate media containing different kinds of peptone and were incubated at 27°, 30°, 35° and 37° C for two days Similar series consisting of one millilitre of each culture were transferred to two or more tubes for VP testing

A comparison was first made of two modifications of Barritt's method Barrit found that an addition of 0 6 ml of a 5 per cent solution of α-naphthol and 0 2 ml of a 40 per cent solution of KOH to 1 ml of culture is as effective as the addition of 0 5 ml of a 6 per cent solution of α-naphthol and 0 5 ml of a 16 per cent solution of KOH Parallel investigations showed that the first modification gives rather more positive reactions than the second The difference is most marked with peptones 2, 3 and 4 at 37° C incubation, the temperature chosen by Barritt for his investigations Barritt's first modification was therefore chosen for the subsequent investigations

In performing the VP test one must pay consideration to the factors which favour the reaction namely agitation and heating Stirring also accelerated the reaction but was not usable in practice On repeated agitation the colour of the reaction becomes darker after each shaking especially in the upper part of the mixture Shaking of the tubes every tenth minute for the first 30 minutes proved to be sufficient The largest number of positive reactions was obtained when the mixtures were left *standing for another 30 minutes* then reshaken and read

To shorten the time before reading of the VP reaction experiments were made with tubes of larger gas absorbing area 20 mm instead of 15 mm diameter Both tubes were shaken every tenth minute for 30 minutes Tubes of 20 mm diameter were read after 30 minutes and 15 mm tubes after 1 hour Table 3 shows that the percentage of positive reactions was greater in 20 mm than in 15 mm tubes All types of peptone at four incubation temperatures gave a larger number of positive reactions in 20 mm tubes This may be explained by the fact that the ingredients were mixed with air more easily and better in 20 mm than in 15 mm tubes

Using the agitation method with tubes of 20 mm diameter the reaction mixture must be shaken every tenth minute *i e* four times in thirty minutes *Heating* was employed to reduce the work involved Serial experiments were made with cultures from 63 coliform strains dispensed in 1 ml portions in two tubes of 20 mm diameter One series was shaken four times in the course of 30 minutes at room temperature and read The other was shaken once placed in incubators at 45° C reshaken after 30 minutes and read The results showed close agreement in cultures grown at 27° C

Comparison with some officially recommended methods of VP testing In view of these investigations it may be said in advance that the techniques prescribed in *The Intern Standards for Drink Water of WHO* (1958) *The Standard Methods of USA* (1960) and *The Bact Ex of Wat Sup* (1956) will not yield satisfactory results The three methods were nevertheless compared with the agitation method All tests were made in tubes of 15 mm diameter The agitation method was found to be superior to the other three methods in respect of the number of positive reactions for all kinds of peptone and all incubation temperatures

Inoculation technique By parallel inoculations with different numbers of bacteria on dextrose phosphate media and titration of 48 hour cultures for acetoin content it was found that the strongest VP reactions occurred after inoculation with a large number of bacteria *e g* 20 million or a full loop of slant agar culture The addition of a low number of bacteria inoculation by once dipping the loop with little material gave positive though weaker reactions

Control of VP reactions by means of acetoin Acetoin (Fairmount Chemical Corp New York 6) and acetyl methyl carbinol (L Leight &

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Co Ltd Colnbrook England) were used for these controls The preparations gave exactly the same results in all tests

A comparison was first made between 1) Voges Proskauer s original method with addition of 5 ml of 40 per cent KOH to 5 ml of culture reading after 24 hours 2) O Meara s method with addition of 25 mg of creatine and 5 ml of 40 per cent NaOH to 5 ml of culture reading after 1 hour and 3) Barritt s method reading 1 hour after the addition of the ingredients (agitation 5 times)

Falling concentrations of both preparations were tested (Table 4) Dilution of acetoin in aq dest 0 5 per cent dextrose or 0 5 per cent K_2HPO_4 gave no reaction Dilutions of 0 5 per cent peptone water (each type of peptone separately) produced reactions by all three methods respectively dextrose K_2HPO_4 and dextrose phosphate media (each type of peptone separately) produced reactions by all three methods The results of these investigations with dextrose phosphate medium are shown in Table 4 Barritt s method was superior to the others acetoin being detected in dilutions of 1/500 000 by Barritt s but only in dilutions of 1 50 000 by the original V P and O Meara s methods *O Meara* (1931) himself stated that his method reveals acetoin in dilution of at most 1 50 000 The reactions were considerably stronger with Barritt s than with the other methods At dilutions of 1/10 and 1/100 the medium developed a yellow colour Dextrose phosphate medium with peptone 2 produced rather weaker reactions in repeated tests which shows that the type of peptone may affect the reaction even without the influence of the bacteria

Comparative tests of the agitation method were made in tubes of 15 and 20 mm diameter as well as of the heating method with different concentrations of the two acetoin preparations The same technique as already recommended was used for these tests The three methods gave exactly the same results as shown in Table 4 for Barritt s method Peptone 2 again showed rather weaker reactions which were not improved even after heating

Titration of dextrose phosphate cultures for acetoin content A study was made of the quantity of acetoin in cultures of 100 V P positive coliform strains grown at 27° C for two days The V P tests were carried out by the heating modification on undiluted cultures and on dilutions of the cultures in aq dest in the proportions 1 2 1 5 1 10 1 20 1 30 and 1 40 Two strains gave positive reactions only in undiluted cultures four up to dilution 1 2 29 up to 1 5 50 up to 1 10 14 up to 1 20 and one strain up to 1 30 The next higher dilutions usually showed ± reactions All strains gave negative reactions in dilution 1 40 The differences in acetoin content had no relation to the ability of the strains to produce gas at 45° C or lower incubation temperature Nor was there any correlation to type of bacteria (A aerogenes intermediate or irregular) nor to origin of strains from water or faeces

A comparison was made between Barritt s and O Meara s methods

TABLΓ 3

Procedure for VP Test Per Cent Positive VP Reactions in 54 Coliform Strains from Water

Incubation temperatures		27°					30°					35°					37°				
Kinds of peptone		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Size of tubes	Read after min																				
15 mm	60	91	82	85	72	91	91	70	83	76	91	89	59	69	63	28	80	41	63	54	65
20 mm	30	94	85	87	76	93	93	76	85	78	93	89	57	72	70	87	78	50	65	57	72

TABLE 4

Comparison of Three Methods for Detection of Acetoin in Dextrose Phosphate Medium

Dilutions of acetoin	Peptone	1 10	1/100	1 1000	1 10 000	1 25 000	1 50 000	1/75 000	1/100 000	1/250 000	1 500 000	1/750 000
Original VP	1–5	yellow	yellow	±	+	+	+	—	—	—	—	—
O'Meara	1 5	yellow	±	+	±	±	±	—	—	—	—	—
Barritt	1	+	+++	+++	+++	+++	+++	++	+	+	±	—
	2	+	+++	+++	+++	+++	++	+	+	±	±	—
	3	+	+++	+++	+++	+++	+++	++	+	+	±	—
	4	+	+++	+++	+++	+++	+++	++	+	+	±	—
	5	+	+++	+++	+++	+++	+++	++	+	+	±	—

+++ — bright raspberry or ruby red
++ — weaker raspberry or ruby red
\+ — aniline or light red
± — very faint pink
— — colourless or copper coloured

Co Ltd, Colnbrook, England) were used for these controls The preparations gave exactly the same results in all tests

A comparison was first made between 1) Voges-Proskauer's original method with addition of 5 ml of 40 per cent KOH to 5 ml of culture, reading after 24 hours, 2) O Meara's method with addition of 25 mg of creatine and 5 ml of 40 per cent NaOH to 5 ml of culture, reading after 1 hour and 3) Barritt's method, reading 1 hour after the addition of the ingredients (agitation 5 times)

Falling concentrations of both preparations were tested (Table 4) Dilution of acetoin in aq dest, 0 5 per cent dextrose or 0 5 per cent K_2HPO_4 gave no reaction Dilutions of 0 5 per cent peptone water (each type of peptone separately) produced reactions by all three methods respectively, dextrose, K_2HPO_4, and dextrose phosphate media (each type of peptone separately) produced reactions by all three methods The results of these investigations with dextrose phosphate medium are shown in Table 4 Barritt's method was superior to the others,

most 1/50,000 The reactions were considerably stronger with Barritt's than with the other methods At dilutions of 1/10 and 1/100 the medium developed a yellow colour Dextrose phosphate medium with peptone 2 produced rather weaker reactions in repeated tests, which shows that the type of peptone may affect the reaction even without the influence of the bacteria

Comparative tests of the agitation method were made in tubes of 15 and 20 mm diameter, as well as of the heating method, with different concentrations of the two acetoin preparations The same technique as already recommended was used for these tests The three methods gave exactly the same results as shown in Table 4 for Barritt's method Peptone 2 again showed rather weaker reactions which were not improved even after heating

Titration of dextrose phosphate cultures for acetoin content A study was made of the quantity of acetoin in cultures of 100 VP positive coliform strains grown at 27° C for two days The VP tests were carried out by the heating modification' on undiluted cultures and on dilutions of the cultures in aq dest in the proportions 1 2, 1 5, 1 10, 1 20, 1 30 and 1 40 Two strains gave positive reactions only in undiluted cultures four up to dilution 1 2, 29 up to 1 5, 50 up to 1 10, 14 up to 1 20, and one strain up to 1 30 The next higher dilutions usually showed ± reactions All strains gave negative reactions in dilution 1 40 The differences in acetoin content had no relation to the ability of the strains to produce gas at 45° C or lower incubation temperature Nor was there any correlation to type of bacteria (A aerogenes, intermediate or irregular), nor to origin of strains from water or faeces

A comparison was made between Barritt's and O'Meara's methods

TABLE 5

Typing of 21 Coliform Strains by Barritt's and O'Meara's Methods

Number of strains	Barritt				O'Meara			
	I	M	V	C	I	M	V	C
4	+	+	+	+	+	+	—	+
4	—	+	+	+	—	+	—	+
5	—	—	+	+	—	—	—	+
4	+	—	+	+	+	—	—	+
1	—	+	+	—	—	+	—	—
2	+	+	+	—	+	+	—	—
1	—	—	+	—	—	—	—	—

in the same culture, 40 strains being tested. All strains which, by Barritt's method, gave positive reactions only in undiluted cultures or in dilutions up to 1 : 2 or 1 : 5, showed negative reactions by O'Meara's method. Only strains which produced a higher acetoin content from dilutions of 1/10 and upwards gave ± and + reactions also by O'Meara's method. In this investigation as well, O'Meara's method proved some ten times less effective than Barritt's.

The effect on the typing result of the use of O'Meara's method compared with Barritt's is shown in Table 5. It is seen that three strains which by O'Meara's method would be typed as Esch. coli did not produce acid and gas at 45° and did not form typical Esch. coli colonies on Endo agar. By Barritt's method, on the other hand, the strains would be regarded as irregular.

DISCUSSION

The purpose of this study has been to evolve a method of performing the VP test which is characterized by the greatest possible measure of standardization and sensitivity. The study confirmed the long known fact that Barritt's method is more sensitive than other generally used methods (*Barritt* 1936, *Levine* 1941, *Batty-Smith* 1941, *Kauffmann* 1954, *Schubert* 1956, *Suassuna et al.* 1960, *The Bacteriological Examination of Water Supplies* 1956, *WHO* 1958, *The Standard Methods of USA* 1960, etc.). According to *The Bacteriological Examination of Water Supplies* and *Taylor, E.* (1958), however, Barritt's method is considered too sensitive, and O'Meara's method, which is ten times less sensitive, is suggested instead. The reason given is that "the high sensitivity of the Barritt's test may tend to obscure differences between members of the coliform group" (*The Bact. Ex.*) and that Esch. coli "may give positive results with the more sensitive reagents" (*Taylor, E.* 1958). The present author has had several reasons for wishing to increase the sensitivity of the reaction. In the first place a sensitive method is desirable for the detection of even small quantities of acetoin in biochemical bacterial examinations. In the second place a sensitive method is preferable also for the typing of coliform bacteria. The production of acetoin is subject to altogether too many varying factors to justify the

necessity for the production of a large quantity as criterion in routine typing

The type of peptone used in the dextrose phosphate medium has a considerably greater rôle in the VP than in the MR test For the choice of the best type of peptone it proved advisable to take several VP-positive strains from faeces and grow them on dextrose phosphate media with different kinds of peptone at up to 45° C It must be remembered, however, that all types of peptone are not universally available and that the quality of the selected peptone may vary On practical grounds it is desirable to use the same medium both for the VP and for the MR tests The type of peptone should therefore be chosen with greater care than has been done previously

The incubation temperature is still an open question Incubation at 27° C was found to give the greatest number of positive VP reactions also with coliform strains from faeces The difference between the peptone varieties is least at 27° C

The present investigations showed two or three days of incubation to *be best for the VP test It is thus possible to carry out VP and MR tests* simultaneously on the third day of incubation, or VP on the second and MR on the fourth day if the third day happens to be a holiday

CONCLUSION

The VP and MR reactions can be tested on the same culture The following medium, reagents and methods are suggested

Medium	Dextrose	5 g
	Peptone	5 g
	K_2HPO_4 (anhydrous)	5 g
	Aq dest	1000 ml

Peptone and K_2HPO_4 are dissolved in aq dest for 20 minutes in steam Dextrose and aq dest are then added to make up 1000 ml The medium is filtered, distributed in 4–5 ml portions into tubes and sterilized in steam, but not for longer than 20 minutes The tubes should not come into contact with the hot condensed water which may collect during the autoclaving The sterility is checked by incubation for 24 hours at 35°–37° C

Reagents 1 0 1 g of methyl red is dissolved in 250 ml 95 per cent ethyl alcohol Alternatively, according to *Clark & Lubs,* 0 1 g of methyl red is dissolved in 300 ml of 95 per cent ethyl alcohol to which 200 ml of aq dest is added

2 5 0 g of α-naphthol is dissolved in 100 ml of 95 per cent ethyl alcohol and kept in a dark flask with ground glass stopper or screw-on cap for not more than 1 month

3 40 per cent KOH solution

Methods

VP test Dextrose phosphate medium is inoculated with a loopful of material and incubated at 27° C After two or three days 1 ml of culture is transferred to a tube of about 20 mm diameter, after which 0 6 ml of α-naphthol solution and 0 2 ml of KOH solution are added The tube is shaken vigorously three times in 30 minutes and the reaction is noted The same result is obtained if the tubes are shaken, incubated at 45° C, reshaken after 30 minutes and read

MR test The rest of the culture is replaced in the incubator at 27° C if the VP test was made after 48 hours of incubation On the next day or two days later, four drops of methyl red solution are added to the culture and the reaction is read within 15 minutes

SUMMARY

A study has been made of the significance of various technical factors on the sensitivity of the VP test In the titration of dextrose phosphate cultures, Barritt's method proved ten times more sensitive than O'Meara's Barritt's method should therefore be employed for the VP test as being the most sensitive

The type of peptone used in the dextrose phosphate medium is of greater significance in the VP than in the MR test Care should therefore be taken in the choice of peptone It is desirable that the medium should be suited for both the VP and the MR test

The best incubation temperature for the VP test is 27° C, *i e* the same temperature as for the MR test The period of incubation for the VP test should be two or three days

The VP reaction can be read 30 minutes after addition of the reagents provided that the mixture is repeatedly shaken in tubes of about 20 mm diameter or is heated to 45° C

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FROM THE DEPARTMENT OF BACTERIOLOGY KAROLINSKA INSTITUTET, STOCKHOLM 60 SWEDEN

A SIMPLE METHOD TO PREPARE PARTICLES IN SUSPENSION FOR ELECTRON MICROSCOPY

By

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Received 7 1 62

A great number of different methods have been developed to prepare particles from suspensions for electron microscopy They range from more exact and troublesome methods, where particular care is taken for the preservation of the three-dimensional structure (*Anderson* 1950, *Williams* 1953), to less subtle but simpler ones, where the suspensions are subjected to air-drying (*e g Valentine & Bradfield* 1954, *Hobbs & Hodgkiss* 1960) We want to describe a simple method not found in recent handbooks (*Anderson* 1956, *Reimer* 1959, *Kay* 1961) which we have now routinely used for over three years

A formwar-coated copper grid (Athene) is taken with a forceps, turned upside down, and the formwar-surface touched to the surface of the suspension to be investigated (usually a faintly turbid suspension, approximately 10^8–10^9 particles/ml, kept in a test tube) When the forceps is raised, a drop of the liquid sticks to the grid The size of this drop is reduced by touching the surface of the drop to the test tube wall or to the surface of the suspension This is most easily achieved when the angle between the grid and the surface to be touched is nearly 90° If the particles to be investigated are suspended in a liquid with high surface tension, *e g distilled water or salt solutions*, the whole drop is often removed and few particles are left on the grid Suspensions with low *surface tension leave an evenly moistened* surface which rapidly dries, and the particles are evenly distributed over the grid Particle suspensions from cells often have low surface tension due to the effect of macromolecules of cellular origin The same effect is achieved by addition of gelatine (final conc 0 01 per cent) (*Williams & Fraser* 1953) A low surface tension of the suspension medium also reduces the flattening of the particles by surface tension forces during drying (*Anderson* 1952) If salts must be present, volatile ones (*e g* ammonium acetate) (*Backus & Williams* 1950) are preferred

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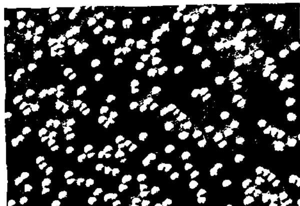


Fig 1 Polystyrene latex spheres (Dow Chemical Co) (× 20 000) Chromium shadowed

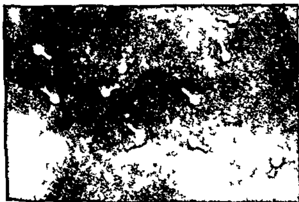


Fig 2 T2 phages (× 24 000 Shadowed with platinum palladium



Fig 3 Cell walls from Streptococcus pyogenes type 12 (×8 000) Chromium shadowed

Ordinary copper grids have routinely been used "Positively charged grids (*Ribi et al* 1960) also give good results but have not been found necessary for our method When examination of the three dimensional structure of the specimen is not so important, the method described above has been preferred, because it is rapid, simple and easily reproduced

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SUPPLEMENT TO THE KAUFFMANN-WHITE-SCHEME (VI)

By

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Received 4 1 63

This paper is the 2 supplement to the *Kauffmann-White-Schema* given in the book *"Die Bakteriologie der Salmonella-Species"* (*F Kauffmann* 1) and the 6 supplement to the review *"Das Kauffmann-White-Schema"* (*F Kauffmann* 2) It contains 79 new *Salmonella species* and 8 variants of known *species* recognized during 1962 From the 79 new *species* 47 belong to *sub genus* I and 32 to *sub genus* II The *species* of *sub-genus* II are indicated by an asterisk (*)

Deviating biochemical results, not given in the table were obtained in the following *species*

**S sofia* fermented salicin after 1 day

**S kuilsrivier* fermented salicin after 1 day and produced indole

S brijbhumi did not ferment maltose and *S uhlenhorst* fermented it delayed and irregularly

**S lincoln* fermented salicin after 4 days

**S sachsenwald* was KCN positive

A culture of *S karamoja* containing the O 1 antigen was found = 1 40 z_{41} 1,2

One culture of *S vleuten* did not ferment salicin and was indole-negative The original culture fermented salicin after 1–2 days and was indole-positive

**S vrindaban* did not ferment sorbitol

**S parera* fermented salicin and maltose after 3 days and was KCN positive This *species* belongs to a special group of *species*, called "atypical *sub genus* II" by *F Kauffmann* (4) in a paper *"Zur Differentialdiagnose der Salmonella Sub Genera I, II und III"* which is in press

To this special, biochemical group belong further the following 6 *species* of Table 1 in the paper *"Supplement to the Kauffmann-White-Scheme (V)"* *S soesterberg* [illegible] *S bonaire* These *species* are [illegible] tive reactions in dulcitol, [illegible] positive reactions in KCN gelatin and salicin, but *S tuindorp* was salicin negative

S soesterberg = 21 $z_4 z_{3}$ – = Ar 2959 55 = Ar 22 1 2 5 6
S houten = 43 $z_4 z_{23}$ – = Ar 1450 53 = Ar 21 1 2 6
S wassenaar = 50 g (p) z_{51} – = Ar \ 99 = Ar 9a 9b 13 1b

Further the following Arizona *species* belongs to these atypical *Salmonella sub genus* II cultures

Ar 396–56 = 10a 10c 13 15 = Sal 40 G –

S abony var haifa = 4 12 b e n x differs from the original *S abony culture* = *4 5 12 b* e n x also biochemically since l tartrate is fermented by *var haifa* promptly by the original culture delayed and irregularly

S sofia var = 4 12 27 b – is closely related to *S sofia* = 4 12 b It is not investigated as yet whether the H antigens are identical or not While *S sofia* fermented d tartrate after 2 days this substance was not fermented by 4 12 27 b –

Further in contrast to *S sofia* 4 12 27 b – did not ferment salicin promptly

S kingston var copenhagen = 4 12 g s t – differs from the original *S kingston* culture = 1 4 12 27 g s t – also biochemically since inositol and l tartrate are fermented promptly by *var copenhagen* by the original culture inositol is not fermented and l tartrate delayed and irregularly

S travis = 4 5 12 g (p) z_1 1 7 *P R Edwards McWhorter & Douglas* who described this *species* identified the special H antigen z_{51} which also is present in *S alamo S maricopa S new mexico* and *S wayne* According to *R Rohde* (personal communication) it is necessary to absorb the g (p) z_1 serum by *S dublin* + *S moscow* + *S budapest* + *S berta* The author has confirmed these results

S lindrick var 1 7 = 9 12 e n x 1 7 This culture which was received from Dr J Taylor London differs from the original *S lindrick* culture – 9 12 e n x 1 5 7 also biochemically since inositol was fermented by the new strain

S rutgers 3 10 l z_{40} 1 7 was omitted from the K W scheme since l z_0 is regarded as an R phase Consequently 2 new types received from Dr R Rohde Hamburg *Salmonella* 3 10 l z_4 1 5 and 9 12 l z_0 1 5 were not added to the K W scheme (in agreement with Dr Rohde)

A new salmonella serotype Salmonella cook (39 z_4 1 5) containing an undescribed flagellar antigen was published by *McWhorter Douglas & Edwards* but not added to the K W scheme by the author since the H antigen z_8 could be in R phase of *S champaign*

Further the following diphasic cultures were isolated

S bleadon = 17 f g t e n x z_1
S wangata – 1 9 12 $z_4 z_{23}$ 1 7
S tunnil = 43 z_4 1 5 7

TABI I

Species	Ara	Dul	Ino	Rha	Tre	Xyl	Cly	H_2S	Gel	d	l	l	Cit	Muc	Mal
•S *sofia* — 4 12 b	+	+		+	+	+	++	+	+	$+^{2}$	—	—	$+^{1\ 2}$	+	+
•S *s flat ar 27* — 4 12 27 b	+	+	—	+	+	+	++	+	+	—	—	—	$+^{2}$	$+^{1}$ -	+
S *abony var haifa* — 4 12 b e n x	+	+	+	+	+	+	++	+	—	+	+	+-	+	$+^{2}$	—
S *mons* — 1 4 12 d l w	+	+		+	+	+	++	+	—	+	+	—	+	$+^{2}$	—
•S *caledon* — 4 12 g m e n x	+	+	—	+	+	+	++	+	+	—	—	—	$+^{1}$ °	+	+
S *kingston var copenhagen* — 4 12 g s t –	+	+	+	+	+	+	$++^{3}$	+	—	+	+	+	+	+	—
•S *durbanville* — 4 12 z_{39} 1 5 7	+	+	—	+	+	+	++	+	+	—	—	—	$+^{2}$	+-	+
•S *california* — 6 7 a z_{42}	+	+	—	+	+	+	++	+	+	—	—	—	+°	+	+
S *rissen* — 6 7 f g	+	+	+	+	+	+	++	+	—	+	+	$+^{2}$	+	+	—
S *ruggil* — 6 7 g t	+	+	+	+	+	+	++	+	—	+	+	—	+	+	—
•S *tulear* — 6 8 a z_{52}	+	+	—	+	+	+	++	+	+	—	—	—	+-	+	+
S *kralingen* — (8) 20 y z_6	+	+	+	+	+	+	++	+	—	—	+	+	+	+	—
S *tarshyne* — 9 12 d 1 6	+	+	—	+	+	+	++	+	—	+	+	—	+	+	—
•S *lindrick var 17* — 9 12 e n x 1 7	+	+	+	+	+	+	++	+	+	×	—	—	+-	+	+
•S *karlsruhe* — 1 9 12 g m s t e n x	+	+	—	+	+	+	—	+	+	—	—	—	+	+	+
S *wangata* — 1 9 12 z_4 z_{23} 1 7	+	+	—	+	+	+	++	+	—	+	×	×	+	+	—
•S — 1 9 12 z_{39} 1 7	+	+	+	+	+	+	++	+	+	—	—	—	+-	+	+
S *anderlecht* — 3 10 c l w	+	+	+	+	+	+	++	+	—	+	+	+	+	+	—
S *maron* — 3 10 d z_{35}	+	+	+	+	+	+	$++^{3}$	+	—	—	+	+	+	+-	—
S *sekondi* — 3 10 e h z_6	+	+	—	+	+	+	++	+	—	+	$+^{2}$	$+^{2}$	+	+	—
•S — 3 15 g m s t –	+	+	+	+	+	+	++	+	+	—	—	—	+-	+	+
S *arkansas* — (3) (15) 34 e h 1 5	+	+	+	+	+	+	++	+	—	+	—	—	+	+	—
S *wildwood* — (3) (15) 34 e h l w	+	+	+	+	+	+	++	+	—	+	+	+	+	+	—
S *menhaden* — (3) (15) 34 l v 1 7	+	+	+	+	+	+	++	+	—	+	×	—	+	+	—
S *broughton* — 1 3 19 b l w	+	+	—	+	+	+	++	+	—	+	+	+-	+	+	—
S — 1 3 19 e h l w	+	+	+	+	+	+	++	+	—	+	+	+	+	+	—
S *maiduguri* — 1 3 19 f g t e n z_{15}	+	+	+	+	+	+	++	+	—	+	+	+°	+	+	—
S *nyor* — 1 3 19 l v 1 5	+	+	+	+	+	+	++	+	—	+	$+^{3}$	—	+	+	—
S *lokstedt* — 1 3 19 l z_{13} z_{28} 1 2	+	+	+	+	+	+	++	+	—	+	+	+	+	+	—
•S *grabouw* — 11 g m s t z_{11}	+	+	—	+	+	+	++	+	+	—	—	—	—	$+^{2}$	+
S *brijbhumi* — 11 i 1 5	+	+	—	—	+	+	—	+	—	$+^{1}$	—	—	—	+	—
S *heerlen* — 11 i 1 6	+	+	—	+	+	+	++	+	—	+	×	—	$+^{2}$	$+^{2}$	—
•S *lincoln* — 11 m t e n x	+	+	+	+	+	+	++	+	+	×	—	—	$+^{2}$	+	+

TABLE (cont.)

Species	Ara	Dul	Ino	Rha	Tre	Xyl	Gly	H_2S	Gel	d	l	i	Cit	Muc	Mal
•S *parera* = 11 z_4 z_{23}	+	—	—	+	+	+	—	+	+	—	—	—	$+^2$	—	—
S *maastricht* = 11 z_{41} 1 2	+	+	—	+	+	+	+ +	+	—	+	+	+	+	+	—
S = 13 22 g m e n z_{15}	+	+	—	+	+	+	+ +	+	—	+	×	—	+	+	—
•S = 13 22 g m t	+	+		+	+	+	+ +	+	+	×	—	—	$+^2$	+	+
•S *goodwood* = 13 22 z_{29} e n x	+	+	—	+	+	+	+ +	+	+	—	—	—	$+^2$	$+^2$	+
S *chagoua* = 1 13 23 a 1 5	+	+	—	+	+	+	+ +	+	—	+	+	+	+	+	–
•S *acres* = 1 13 23 b z_{42}	+	+	—	+	+	+	+ +	+	+	×	—	—	$+^2$	+	+
S *rutten* = 13 23 d l w	+	+	+	+	+	+	+ +	+		+	$+^2$	$+^2$	+	+	—
S *isuge* = 13 23 d z_6	+	+	—	+	+	+	+ +	+	—	+	+	+	+	+	—
S *congo* = 13 23 g t –	+	+	+	+	+	+	+ +	+	+	+	+	$+^{1}$ –	+	+	—
•S *gojenberg* = 1 13 23 g t 1 5	+	+	—	+	+	+	+ $+^3$	+	+	×	—	—	$+^2$	$+^2$	+
•S *stevenage* = 1 13 23 z_{42} 1 7	+	+	—	+	+	+	+ +	+	+	×	—	—	$+^2$	+	+
S *finkenwerder* = 1 6 14 25 d 1 5	+	+	+	+	+	+	+ +	+	—	+	+	$+^2$	+	+	—
S *schallwijk* = (6) 14 (24) i e n	+	+	—	+	+	+	+ +	+	–	+	×	—	+	+	—
S *kaitaan* = 1 6 14 25 m t –	+	+	—	+	+	+	+ +	+	—	$+^3$	$+^2$	—	+	+	—
S *fischerhuette* = 16 a e n z_{15}	+	+	—	+	+	+	—	+		+	—	—	$+^2$	—	—
S *heron* = 16 a z_6	+	+	+	+	+	+	+ +	+	—	+	+	+	+	+	—
S *barmbek* = 16 d z_3	+	+	+	+	+	+	+ +	+	—	+	×	$+^2$	+	+	—
S *burgas* = 16 l v e n z_{15}	+	—	—	+	+	+	+ +	+	—	+	$+^2$	—	+	—	—
•S *bleadon* = 17 f g t e n x z_{15}	+	$+^2$	—	+	+	+	+ +	+	+	×	—	—	$+^2$	+	+
•S *beloha* = 18 z_{36}	+	+	—	+	+	+	+ +	+	+	—	—	—	$+^2$	$+^2$	+
S *assen* = 21 a	+	+	—	+	+	+	+ +	+	—	+	×	$+^2$	+	+	—
S = 21 c e n x	+	+	—	+	+	×	+ $+^5$	+	—	+	×	—	$+^2$	+	—
S *wedding* = 28 c e n z_{15}	+	+	—	+	+	+	+ +	+	–	+	—	—	+	+	—
S *massakory* = 35 r l w	+	+	+	+	+	+	+ +	+	—	+	+	+	+	+	—
S *ligna* = 35 z_{10} z_6	+	+	+	+	+	+	+ +	+	—	+	+	+	+	+	—
•S *carletonville* = 38 d	+	+	—	+	+	+	+ +	+	+	×	—	—	+	+	+
S *santhiaba* = 40 l z_{28} 1 6	+	+	—	+	+	+	+ +	+	—	+	+	—	+	+	—
•S *sachsenwald* = 1 40 z_4 z_{23}	+			+	+	$+^3$		+	+	—	—	—	+–	—	—
•S *grunty* = 1 40 z_{39} 1 6	+		—	+	+	+	+ +	+	+	$+^2$	—	—	$+^2$	+	+
S *karamoja* = 1 40 z_{41} 1 2	+	+	+	+	+	+	+ +	+	—	+	+	+	+	+	—
S *inpraw* = 41 z_{10} e n x	+	+	+	+	+	+	+ +	+		+	+	+	+	+	—
S *harvestehude* = 1 42 y z_6	+	+	+	+	+	+	+ +	+	—	+	+	$+^2$	+	+	—
•S *bunnik* = 43 z_{42} 1 5 7	+	+	—	+	+	+	+ +	+	+	×	—	—	$+^2$	+	+

TABLE (cont)

Species	Ara	Dul	Ino	Rha	Tre	Xyl	Gly	H_2S	Gel	d	l	i	Cit	Muc	Mal
S wright — 43 z_{29}	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S vleuten — 44 f g	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
S uhlenhorst — 44 z l w	+	+	+	+	+	+	++	+	—	+	+	+2	+	+	—
**S rinfaban* — 45 a e n x	+	—	—	+	+	+	++	+	+	×	—	—	+-	+	—
**S ejeda* — 45 a z_{10}	+	+	—	+	+	+	++	+	+	—	—	—	+3	+	+
S suelldorf — 45 f g	+	+	—	+	+	+	++	+	—	+	+	—	+	+	—
S — 45 g m	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
**S bremen* — 45 g m s t e n x	+	+	—	+	+	+	++	+	+	—	—	—	+-	+2	+
**S perinet* — 45 m t e n x z_{15}	+	+	—	+	+	+	++	+	+	—	—	—	×	+	+
**S klapmuts* — 45 z z_{39}	+	+	—	+	+	+	++	+	+	×	—	—	+2	+2	+
S stellingen — 47 d e n x	+	+	—	+	+	+	++3	+	—	+	—	—	+2	+	—
S moualine — 47 y 1 6	+	+	—	+	+	+	++	+	—	+	×	—	+	+	—
**S chersina* — 47 z z_6	+	+	—	+	+	+	++	+	+	×	—	—	+2	+1 2	+
**S hammonia* — 48 e n x z_{15} z_1	+	—	—	+	+	+	++	+	+	—	—	—	+2	+	+
**S hooggraven* — 50 z_{10} z_6 z_{42}	+	+	—	+	+	+	++	+	+	×	—	—	+2	+2	+
S meskin — 51 e h 1 2	+	+	+	+	+	+	++	+	—	+	+	+	+	+	-
**S* — 51 - 1 7	+	+	—	+	+	+	++	+	+	—	—	—	+2	+	+
**S manombo* — 57 z_{39} e n x z_{15}	+	+	—	+	+	+	++	+	+	—	—	—	+2	+	+
**S beltoky* — 59 k (z)	+	+	—	+	+	+	++	+	+	—	—	—	+2	+	+
**S luton* — 60 z e n x	+	+	—	+	+	+	++	+	+	—	—	—	+2	+	+

Key Ara — arabinose Dul — dulcitol Ino — inositol Rha — rhamnose, Tre — trehalose Xyl — xylose Gly — Stern's glycerol fuchsin broth Gel — gelatin d — d tartrate l — l tartrate, i — i tartrate Cit — sodium citrate, Muc — mucate Mal — sodium malonate Arabinose to xylose + — positive after 1 day — — negative after 30 days × — late and irregularly positive or negative Stern's glycerol fuchsin broth ++ lilac after 1 2 days, — negative after 8 days Gelatin + slowly positive in ferrous chloride gelatin and rapidly positive in the Kohn test modified by Lautrop Organic acids + — positive after 1 day +2 — positive after 2 3 days — — negative after 14 days * — *sub genus* II

With regard to the new *Salmonella* O groups the following should be mentioned

Salmonella O 50 identical with Arizona 9a,9c
Salmonella O 51 identical with Arizona 1,2
Salmonella O 52 identical with Arizona 31
Salmonella O 53 related to Arizona 1,4
Salmonella O 56 identical with Arizona 14
Salmonella O 57 identical with Arizona 34
Salmonella O 58 identical with Arizona 1,33
Salmonella O 59 related to Arizona 19
Salmonella O 60 identical with Arizona 24

List of Salmonella Species Recognized in 1962

Salmonella abony var haifa Kauffmann = 4,12: b e,n,x
* *Salmonella acres* = 1,13,23 b z_{10}
Salmonella anderlecht van Oye, Fievez, Granville & van Goethem = 3,10 e l,w Annal Inst Pasteur *103* 931–932, 1962
Salmonella arkansas = (3),(15),34 e,h 1,5
Salmonella assen = 21 a –
Salmonella barmbek Rohde & Bischoff = 16 d z_6 Z Hyg in press
* *Salmonella beloha nom nov Kauffmann* (Salmonella beloha Le Minor, Le Noc & Coynault) = 18 z_{36} – Bull Soc Path exot *55* 216–220, 1962
* *Salmonella betioky nom nov Kauffmann* (Salmonella betioky Le Minor, Le Noc & Coynault) = 59 k (z) Bull Soc Path exot *55* 216–220, 1962
* *Salmonella bleadon*, diphasic = 17 f,g,t e,n,x,z_{15}
* *Salmonella bremen* = 45 g m,s,t e,n,x (45_1,45_2)
Salmonella brijbhumi = 11 i 1 5
Salmonella broughton = 1,3,19 b l,w
* *Salmonella bunnik*, diphasic = 43 z_{10} 1,5,7 (43_1,43_3,43_4)
Salmonella burgas = 16 l,v e,n,z_{15}
* *Salmonella caledon Brede* = 4,12 g,m e,n,x Annal Inst Pasteur *103* 933–934, 1962
* *Salmonella calvinia* = 6,7 a z_{42}
* *Salmonella carletonville* = 38 d
Salmonella chagoua = 1,13,23 a 1,5
* *Salmonella chersina* = 47 z z_6 (47_1,47_3)
Salmonella congo = 13,23 g,t
* *Salmonella durbanville* = 4,12 z_{39} 1,5,7
* *Salmonella ejeda nom nov Kauffmann* (Salmonella ejeda Le Minor, Le Noc & Coynault) = 45 a z_{10} (45_1,45_3) Bull Soc Path exot *55* 216 220 1962

Salmonella finkenwerder Rohde & Tiedje = 1 6 14 25 d 1 5
Z Hyg in press
Salmonella fisel erhuette — 16 a e n z15
Salmonella gojenberg Rohde & Muller = 1 13 23 g t 1 5
Z Hyg in press
Salmonella goodwood — 13 22 z29 e n x
Salmonella grabouw — 11 g m s t z39
Salm nella grunty = 1 40 z39 1 6 (1 40₁ 40₃)
Salmonella hammonia Rohde & Muller = 48 e n x,z15 z (48₁ 48)
Z Hyg in press
Salmonella harvestehude Rohde & Tiedje = 1 42 y z6
Z Hyg in press
Salmonella heerlen — 11 i 1 6
Salmonella heron — 16 a z
Salmonella hooggraven — 50 z10 z6 z4 (51₁ 50₃)
Salmonella inpraw = 41 z10 e n x
Salmonella irigny = 43 z 5 (43₁ 43)
Salmonella isuge = 13 23 d z6
Salmonella kattaan = 1 6 14 25 m t
Salmonella karamoja with O 1 antigen — 1 40 z41 1 2 (1 40₁ 40)
Salmonella kingston var copenhagen Kauffmann — 4 12 g s t
Salmonella klapmuts = 45 z z39 (45₁ 45₂)
Salmonella kralingen — (8) 20 y z6
Salmonella kuilsrivier — 1 9 12 g m s t e n x
Salmonella ligna — 35 z10 z6
Salmonella lincoln = 11 m t e n x
Salm nella lin trick var 1 — 9 12 e n x 1 7
Salmonella lokstedt Rohde & Tiedje — 1 3 19 l z 3 z 5 1 2
Z Hyg in press
Salmonella lul a — 60 z e n x
Saln onella m iastricht — 11 z41 1 2
Salm nella ma lu juri Rohde & Tiedje — 1 3 19 f g t e n,z 5
Z Hyg in press
Salmonella manombo nom nov Kauffmann
(Salmonella manomb o Le Minor Le Noc & Coynault) —
5 z39 e n x z 5 Bull Soc Path exot 55 216–220 1962
Salmonella maron 3 10 d z3
Salm nella massak ry = 35 r l w
Salmor ella menha len = (3) (15) 34 l z 1 7
Salm nella meskin = 51 e h 1 2
Salm nella mons i an Ope & Nechelput = 1 4 12 d l w
Ann d Inst Pasteur in press
Salm nella m naline = 47 x 1 6 (47₁ 47₃)
Salm nella ngor nom nov Kauffmann
(Salmonella ngor Le Minor Chamb on Bories Marx & Charié-Mar saines) — 1 3 19 l v 1 Bull Soc Path exot 55 213–215 1962

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Salmonella brijbhumi = 11 i 1,5
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**Salmonella bunnik*, diphasic = 43 z_{42} 1,5,7 (43_1,43_3,43_4)
Salmonella burgas = 16 l,v e,n,z_{15}
**Salmonella caledon Brede* = 4,12 g,m e,n,x Annal Inst Pasteur *103* 933–934, 1962
**Salmonella calvinia* = 6,7 a z_{40}
**Salmonella carletonville* = 38 d –
Salmonella chagoua = 1,13,23 i 1,5
**Salmonella chersina* = 47 z z_6 (47_1,47_3)
Salmonella congo = 13,23 g,t –
**Salmonella durbanville* = 4,12 z_{39} 1,5,7
**Salmonella ejeda nom nov Kauffmann* (Salmonella ejeda Le Minor, Le Noc & Coynault) = 45 a z_{10} (45_1,45_3) Bull Soc Path exot *55* 216 220 1962

Salmonella finkenwerder Rohde & Tiedje — 1 6 14 25 d 1 5 Z Hyg in press
Salmonella fischerhuette — 16 a e n z_{15}
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Salmonella goodwood — 13 22 z_0 e n x
Salmonella grabouw = 11 g m s t z_{39}
Salmonella grunty — 1 40 z_{39} 1 6 (1 40_1 40_3)
Salmonella hammonia Rohde & Muller — 48 e n x z_{15} z_6 (48_1 48) Z Hyg in press
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Salmonella heerlen — 11 i 1 6
Salmonella heron — 16 a z_6
Salmonella hooggraven — 50 z_0 z_6 z_4 (51_1 50_3)
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Salmonella irigny — 43 z_{38} (43_1 43)
Salmonalla isuge — 13 23 d z_6
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Salmonella kralingen — (8) 20 y z_6
Salmonella kuilsrivier = 1 9 12 g m s t e n x
Salmonella ligna — 35 z_0 z_6
Salmonella lincoln = 11 m t e n x
Salmonella lindrick var 1 9 12 e n x 1 7
Salmonella lokstedt Rohde & Tiedje = 1 3 19 l z_{13} z_{28} 1 2 Z Hyg in press
Salmonella luton 60 z e n x
Salmonella maastricht — 11 z_{41} 1 2
Salmonella malluguri Rohde & Tiedje = 1 3 19 f g t e n z Z Hyg in press
Salmonella manombo nom nov Kauffmann (*Salmonella manombo* Le Minor Le Noc & Coynault) — 57 z_5 e n x z_{15} Bull Soc Path exot 55 216–220 1962
Salmonella maron — 3 10 d z_3
Salmonella massakory = 35 r l w
Salmonella menhaden = (3) (15) 34 l v 1 7
Salmonella meskin = 51 e h 1 2
Salmonella minnesota van Oye & Nechelput = 1 4 12 d l w Annal Inst Pasteur in press
Salmonella moualine = 47 y 1 6 (47_1 47_3)
Salmonella ngor nom nov Kauffmann (*Salmonella ngor* Le Minor Chambon Bories Marx & Charié Marsaines) = 1,3 19 l v 1 5 Bull Soc Path exot 55 213 215 1962

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Bull Soc Path exot *55* 216 220, 1962
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(Salmonella ejeda Le Minor, Le Noc & Coynault) = 45 a z_{10}
(45_1,45_3) Bull Soc Path exot *55* 216 220, 1962

Addendum to Supplement V
(Kauffmann 3)

Salmonella gateshead — (9) 46 g s t
Salmonella jukestown = 13 23 i e n z_1
Salmonella lobatsi = 52 1 5 7
Salmonella tejas nom nov Kauffmann
(Salmonella tejas Heather Howard Dag & Bredthauer) — 4 12 z_{36} –
J Bacter *84* 377 1962
Salmonella travis nom nov Kauffmann
(Salmonella travis Edwards McWhorter & Douglas) —
4 5 12 g (p),z t 1 7 Internat Bull Bact Nomencl & Taxon
12 67 1962
Salmonella wingrove = 6 8 c 1 2
Salmonella bonaire was written by a mistake without the e
Salmonella zeist = 18 z_{10} z_6 belongs to *sub genus* II

SUMMARY

A supplement to the *Kauffmann White Scheme* is given containing 79 new *Salmonella species* and 8 variants recognized during the year 1962

REFERENCES

Edwards P R McWhorter A C & Douglas G W A new Salmonella serotype Salmonella travis (4 5 12 g (p) z 1 1 7) and a redefin tion of the H antigens of several Salmonella serotypes Internat Bull Bacter Nomenclat & Taxon *12* 67 0 1962

Kauffmann F (1) D e Bakter olog e der Salmonella Spec es (Munksgaard Copenhagen 1961)

Kauffmann F (2) Das Kauffmann White Schema n Ergebnisse der M krobio

Kau

Kau

McW

Internat Bull Bacter Nomenclat & Taxon *12* 181 183 1962

**Salmonella parera* = 11 z_1,z_{23} –
**Salmonella perinet nom nov Kauffmann*
(Salmonella perinet Le Minor, Le Noc & Coynault) =
45 m,t e,n,x,z_{15} ($45_1,45_2,45_3$) Bull Soc Path exot 55 216 220
1962
Salmonella putten = 13,23 d l,w
Salmonella riggil = 6,7 g,t –
Salmonella rissen Rohde & Tiedje = 6,7 f,g –
Z Hyg in press
**Salmonella sachsenwald Rohde & Muller* = 1,40 z_1,z_{23} – ($40_1,40_2,40_3$)
Z Hyg in press
Salmonella santhiaba nom nov Kauffmann
(Salmonella santhiaba Le Minor, Chambon, Bories, Marx & Charie Marsaines) = 40 l,z_{28} 1,6 ($40_1,40_2$) Bull Soc Path exot 55 213-215, 1962
Salmonella schalkwijk = (6),14,(24) i e,n
Salmonella sekondi = 3,10 e,h z_6
**Salmonella sofia Wesselinoff & Dimow* = 4,12 b
Zbl Bakter I Orig *187* 263 265, 1962
**Salmonella sofia var 27* = 4,12,27 b –
Salmonella stellingen Rohde & Bischoff = 47 d e,n,x ($47_1,47_3$)
Z Hyg in press
**Salmonella stevenage* = 1,13,23 z_{42} 1,7
Salmonella suelldorf Rohde & Weissflog = 45 f,g ($45_1,45_3$)
Z Hyg in press
Salmonella tarshyne = 9,12 d 1,6
**Salmonella tulear* = 6,8 a z_{52}
Salmonella uhlenhorst Rohde, Bischoff & Tiedje = 44 z l,w
Z Hyg in press
Salmonella vleuten = 44 f,g –
**Salmonella vrindaban* = 45 a e,n,x ($45_1,45_3$)
Salmonella wangata = 1,9,12 z_4,z_{23} 1,7
Salmonella wedding = 28 c e,n,z_1
Salmonella wildwood = (3),(15),34 e,h l,w
**Salmonella* 1,9,12 z_{39} 1,7
**Salmonella* 3,15 g,m,s,t
Salmonella 1,3,19 e,h l,w
Salmonella 13,22 g m e,n,z_1
**Salmonella* 13,22 g,m,t –
Salmonella 21 c e,n,x
Salmonella 45 g,m – (45_1 45)
**Salmonella* 51 – 1,7

Die Sonderstellung des *sub genus* II kommt auch bei der Verteilung der H Antigene zum Ausdruck, da besonders haufig folgende H Antigen Komplexe vertreten sind diphasische G Komplexe wie g,m,s,t z_{12} oder g,m,s,t z_{39} oder f g t e,n,x,z_{15} etc, die H-Antigene z, z_{39} und z_{42} sowie die 2 Phasen 1, 5, 7 und e,n,x,z_{15} Bisher sind die H Antigene z_{39} und z_{42} sowie 1,5,7 und e,n,x,z_{15} noch nicht bei *species* des *sub-genus* I gefunden *S hisingen* = 48 a 1,5 7 ist eine intermediare *species* und konnte zum *sub-genus* II gerechnet werden, obwohl sie Gelatine nicht verflussigt

Die 2 Phasen 1, 5, 7 und e,n,x,z_{15} kommen wiederholt im *sub-genus* III(Arizona) vor, doch sind die H-Antigene z_{39} und z_{42} bisher noch nicht in diesem *sub genus* gefunden worden Die 3 *sub genera* haben also eine charakteristische Antigenverteilung, die weitere Beachtung verdient

So ist z B damit zu rechnen, dass das H Antigen z, welches komplex gebaut ist und im *sub genus* II sehr haufig vorkommt, von den z Antigenen des *sub genus* I verschieden ist Das entsprechende gilt auch fur andere H Antigene Wahrend es also mit Rucksicht auf die bakterio [illegible] h [illegible] zieren bestimmt man die e,n,x z_{15}-Phase nur nach dem vereinfachten K W-Schema mit e,n, so ubersieht man, dass diese e,n Phasen (= e n,x, z_{15}) im *sub genus* I uberhaupt nicht vorkommen Deshalb wissen wir heute nicht, ob die zahlreichen z-Antigene in den *sub genera* I und II identisch sind oder nicht

Die Grenzen zwischen den *sub genera* I, II und III sind, wie bei allen biochemischen Gruppen, nicht scharf, sodass es in einigen Fallen willkurlich ist, wohin man eine *species* rechnen will Wahrend also die *species* Diagnose scharf ist, so ist die *sub genus* Diagnose unscharf Alle diejenigen *species*, die, wie z B *S schleissheim*, Gelatine verflussigen, sich aber in den organischen Säuren wie Mitglieder des *sub genus* I verhalten, stehen zwischen I und II Andere *species*, die Gelatine verflussigen und Dulcit nicht spalten, stehen zwischen II und III

In epidemiologischer Hinsicht haben alle weiteren Erfahrungen die fruhere Feststellung bestätigt, dass die überwiegende Mehrzahl der *sub-genus* II-*species* aus Sudafrika stammt Sie sind teils aus Menschen oder Tieren und teils aus Fischmehl, Eiprodukten oder anderen Nahrungsmitteln, die aus Afrika importiert wurden, isoliert

Da die Zahl der *species* im *sub-genus* I ca 700 und im *sub genus* II ca 130 betragt so kommen wir auf ca 1000 *Salmonella species*, wenn wir das *sub-genus* III (Arizona) hinzurechnen Da ferner die wichtigsten und häufigsten *species* zu den O Gruppen A–E des *sub genus* I gehoren, so ist es berechtigt, weitere *species* der *sub genera* II und III nur nach dem vereinfachten K-W-Schema zu diagnostizieren

ZUR SEROLOGIE DES SALMONELLA SUB GENUS II

Von

F KAUFFMANN

Eingegangen 15 i 63

Das *Salmonella sub-genus* II wurde 1960 vom Verfasser in seiner Mitteilung *„Two biochemical sub-divisions of the genus Salmonella"* aufgestellt und enthielt 55 *species* Auf Grund eines Vorschlages von *F Kauffmann & R Rohde* sollen weitere *species* dieses *sub-genus* II ab 1963 nur nach dem vereinfachten *Kauffmann-White-Schema* (K-W-Schema) diagnostiziert werden

Es erschien deshalb wunschenswert, alle bisher bekannten 131 *species* dieses *sub-genus* II in einer Tabelle zusammenzustellen, um einen Überblick uber die serologischen Besonderheiten zu erhalten Über die biochemischen Reaktionen des *sub-genus* II wurde in einer vorhergehenden Mitteilung des Verfassers *„Zur Differentialdiagnose der Salmonella sub-genera I, II und III"* berichtet, sodass hierauf verwiesen sei

Die in der folgenden Tabelle nicht aufgefuhrten ca 700 *species* des *sub-genus* I sind in dem Kapitel *„Das Kauffmann-White-Schema*" in dem Buche *„The World Problem of Salmonellosis"* (herausgegeben von Dr *E van Oye*) angegeben Ferner sei auf das Buch des Verfassers „*Die Bakteriologie der Salmonella Species"* verwiesen sowie auf die beiden Supplemente V und VI zum K-W-Schema

Bis Ende 1962 waren im ganzen uber 830 *Salmonella species* der *sub genera* I und II bekannt, hiervon gehoren 131 zum *sub-genus* II und ca 700 *zum sub-genus* I Vergleichen wir die O Gruppen-Verteilung in beiden *sub genera*, so sehen wir, dass die klassischen O-Gruppen A–E beim *sub genus* II nur in ca 30 %, beim *sub genus* I aber in ca 60 % vorkommen Die hoheren O Gruppen treten also im *sub genus* II erheblich haufiger als im *sub-genus* I auf

Hierbei ist zu berucksichtigen, dass es sich nur um die Gesamtzahl der *species* handelt, nicht aber um ihre Haufigkeit im pathologischen Materiale Berucksichtigen wir diese, so kommen beim *sub-genus* I hauptsachlich *species* der O-Gruppen A–E vor wahrend beim *sub-genus* II die *species* der hoheren Gruppen sehr stark uberwiegen

Gehen wir weiter zum *sub-genus* III (Arizona) so sind die hoheren O Gruppen noch haufiger als im *subgenus* II vertreten, sodass also dieses *sub genus* II auch in serologischer Hinsicht eine Zwischenstellung zwischen den *sub-genera* I und III einnimmt

Species	Körper-Antigene	Geissel-Antigene	
		1 Phase	2 Phase
S westpark	3 10	l z_{13}	e n,x
S alexander	3 10	z	1,5
S finchley	3 10	z	e n,x
S mp la	3 10	z_{38}	z_{42}
S winchester	3 10	z_{39}	1 7
Gruppe F 2			
S	3 15	g m s t	–
Gruppe F			
S montgomery	11	d(a)	d e n z_{15}
S grabouw	11	g m s t	z_{39}
S line ln	11	m t	e n,x
S huila	11	l z_{28}	e n,x
S parera	11	z_4,z_{23}	–
Gruppe G 1			
S	13 22	g m t	–
S rotterdam	1 13,22	g t	1 5
S elfton	13,22	z_{29}	1 5
S goodwood	13,22	z_{29}	e,n x
Gruppe G 2			
S acres	1 13 23	b	$z_{4?}$
S luanshya	13 23	g s (t)	–
S gojenberg	1 13 23	g t	1 5
S katesgrove	1 13 23	m t	1 5
S worcester	1 13.23	m t	e n,x
S nachshonim	1 13.23	z	1 5
S stevenage	1 13.23	z_{42}	1 7
Gruppe I			
S bellville	16	e n,x	1 7
S moheni	16	g m s t	–
S merseyside	16	g t	1 5
S rowbarton	16	m t	
S haldon	16	z_4,z_{23}	
S jacksonville	16	z_{29}	–
S woodstock	16	$z_{4?}$	1 (5) 7
S elsiesrivier	16	$z_{4?}$	1 6
Gruppe J			
S hillbrow	17	b	e n,x,z_{15}
S verity	17	e n x,z_{15}	1 6
S bleadon	17	f g t	[e n x,z_{15}]
Gruppe K			
S zeist	18	z_{10}	z_6
S belha	18	z_{36}	–

KAUFFMANN-WHITE-SCHEMA

Sub genus II

Species	Körper-Antigene	Geissel-Antigene	
		1. Phase	2. Phase
Gruppe B			
S. sofia	4, 12	b	–
S. sofia var. 27	4, 12, 27	b	–
S. makumira	4, 12	e, n, x	1, 7
S. caledon	4, 12	g, m	e, n, x
S. bechuana	4, 12, 27	g, t	–
S. kilwa	4, 12	l, w	e, n, x
S. nordenham	1, 4, 12, 27	z	e, n, x
S. durbanville	4, 12	z_{39}	1, 5, 7
Gruppe C 1			
S. calvinia	6, 7	a	z_4
S. bloemfontein	6, 7	b	z_{42}
S. heilbron	6, 7	l, z_{28}	1, 5
S. tosamanga	6, 7	z	1, 5
S. baccongo	6, 7	z_{36}	z_{42}
S. gilbert	6, 7	z_{39}	1, 7
S. sullivan	6, 7	z_{42}	1, 7
Gruppe C 2			
S. tulear	6, 8	a	z_{52}
S. baragwanath	6, 8	m, t	1, 5
S. germiston	6, 8	m, t	e, n, x
Gruppe D 1			
S. mjimwema	1, 9, 12	b	e, n, x
S. blankenese	1, 9, 12	b	z_6
S. zuerich	1, 9, 12	c	z_{39}
S. lindrick	9, 12	e, n, x	1, 5, 7
S. lindrick var. 1, 7	9, 12	e, n, x	1, 7
S. kuilsrivier	9, 12	g, m, s, t	e, n, x
S. manica	1, 9, 12	g, m, s, t	z_{42}
S. neasden	9, 12	g, s, t	e, n, x
S. hamburg	1, 9, 12	g, t	
S. dar es salaam	1, 9, 12	l, w	e, n, x
S. stellenbosch	1, 9, 12	z	1, 7
S. angola	1, 9, 12	z	z_6
S. hueningen	9, 12	z	z_{39}
S. canastel	9, 12	z_{29}	1, 5
S.	1, 9, 12	z_{39}	1, 7
Gruppe D 2			
S. lundby	(9), 46	b	e, n, x
S. haarlem	(9), 46	z	e, n, x
Gruppe E 1			
S. chudleigh	3, 10	e, n, x	1, 7
S. islington	3, 10	g, t	–
S. fuhlsbuettel	3, 10	l, v	z_6

Species	Körper-Antigene	Geissel Antigene	
		1 Phase	2 Phase
Gruppe W			
S vrindaban	45	a	e n,x
S ejeda	45	a	z_{10}
S bremen	45	g m s t	e n,x
S windhoek	45	g t	1,5
S perinet	45	m t	e n,x,z_{15}
S klapmuts	45	z	z_{39}
Gruppe X			
S bilthoven	47	a	–
S phoenix	47	b	1 5
S quimbamba	47	d	z_{39}
S chersina	47	z	z_6
Gruppe Y			
S hammonia	48	e n,x z_{15}	z_6
S sakaraha	48	k	z_{39}
S ngozi	48	z_{10}	1,5
Gruppe Z			
S krugersdorp	50	e n x	1 7
S wassenaar	50	g (p) z_{51}	–
S greenside	50	z	e n,x
S bonaire	50	z_4,z_{32}	–
S hooggraven	50	z_{10}	z_6 z_{42}
Gruppe 51			
S	51	–	1 7
Gruppe 52			
S 1 batsi	52	–	1,5 7
Gruppe 53			
S midhurst	53	l z_{28}	z_{39}
S humber	53	z_4 z_{24}	–
Gruppe 55			
S tranoroa	55	k	z_{39}
Gruppe 56			
S artis	56	b	–
Gruppe 57			
S locarno	57	z_{29}	z_{42}
S manombo	57	z_{39}	e n,x,z_{15}

Species	Körper Antigene	Geissel Antigene	
		1 Phase	2 Phase
Gruppe L			
S soesterberg	21	z_4 z_{23}	
S gwaai	21	z_4 z_{24}	–
S wandsbek	21	z_{10}	z_6
Gruppe M			
S kaltenhausen	28	b	z_6
S ceres	28	z	z_{39}
Gruppe P			
S carletonville	38	d	–
S foulpointe	38	g t	–
Gruppe Q			
S mondeor	39	l z_{28}	e n x
Gruppe R			
S springs	40	a	z_{39}
S suarez	1 40	c	e n x z_{15}
S alsterdorf	1 40	g m t	–
S boksburg	40	g s	e n x z_{15}
S bulawayo	1 40	z	1 5
S sachsenwald	40	z_4 z_{23}	–
S degania	40	z_4 z_{24}	–
S bern	1,40	z_4 z_{32}	–
S fandran	1,40	z_{35}	e n x z_{15}
S grunty	1 40	z_{39}	1 6
Gruppe S			
S lethe	41	g t	
S negev	41	z_{10}	1 2
S lichtenberg	41	z_{10}	z_6
Gruppe T			
S chinovum	42	b	1 5
S uphill	42	b	e n x z_{15}
S fremantle	42	(f) g t	
S portbech	42	l v	e n x z_{15}
S nairobi	42	r	
S detroit	42	z	1 5
S rand	42	z	e n x z_{15}
Gruppe U			
S houten	43	z_4 z[illegible]	
S tuindorp	43	z_4 z[illegible]	
S bunnik	43	z_4[illegible]	[1 5 7]

Species	Körper-Antigene	Geissel Antigene	
		1 Phase	2 Phase
Gruppe W			
S vrindaban	45	a	e n x
S ejeda	45	a	z_{10}
S bremen	45	g m s t	e n,x
S windhoek	45	g t	1,5
S perinet	45	m t	e n,x z_{15}
S klapmuts	45	z	z_{39}
Gruppe X			
S bilthoven	47	a	–
S phoenix	47	b	1 5
S quimbamba	47	d	z_{39}
S chersina	47	z	z_6
Gruppe Y			
S hammonia	48	e n,x z_{15}	z_6
S sakaraha	48	k	z_{39}
S ngozi	48	z_{10}	1 5
Gruppe Z			
S krugersdorp	50	e n x	1 7
S wassenaar	50	g (p),z_{51}	–
S greenside	50	z	e n x
S bonaire	50	z_4 z_{24}	–
S hooggraven	50	z_{10}	z_6 z_{42}
Gruppe 51			
S	51	–	1 7
Gruppe 52			
S lobatsi	52		1,5 7
Gruppe 53			
S midhurst	53	l z_{28}	z_{39}
S humber	53	z_4,z_{24}	–
Gruppe 55			
S tranoroa	55	k	z_{39}
Gruppe 56			
S artis	56	b	–
Gruppe 57			
S locarno	57	z_{29}	z_{42}
S manombo	57	z_{39}	e n x,z_{15}

Species	Körper Antigene	Geissel Antigene	
		1 Phase	2 Phase
Gruppe 58			
S basel	58	l z_{13} z_{28}	1 5
Gruppe 59			
S betioky	59	k	(z)
Gruppe 60			
S luton	60	z	e n x

ZUSAMMENFASSUNG

Es werden die Antigenformeln der bisher bekannten 131 *Salmonella species* des *sub-genus* II in einer Tabelle zusammengestellt und mit den ca 700 *species* des *sub-genus* I verglichen

Zu den O-Gruppen A–E gehoren nur ca 30 % der *species* des *sub genus* II, aber ca 60 % derjenigen des *sub genus* I Beim *sub genus* II kommen also uberwiegend die hoheren O-Gruppen vor, sodass dieses *sub-genus* auch in serologischer Hinsicht eine Zwischenstellung zwischen den *sub genera* I und III (Arizona) einnimmt

Auch die H-Antigen-Verteilung innerhalb der 3 *sub-genera* ist deutlich verschieden, sodass diese einen charakteristischen Antigenaufbau besitzen

LITERATUR

Kauffmann F Two biochemical sub divisions of the genus Salmonella Acta path et microbiol scandinav *49* 393 396 1960

Kauffmann F Die Bakteriologie der Salmonella Species Munksgaard Kopenhagen *1961*

Kauffmann F Supplement to the Kauffmann White Scheme (V) Acta path et microbiol scandinav *55* 349 354 1962

Kauffmann F Supplement to the Kauffmann White Scheme (VI) Acta path et microbiol scandinav im Druck

Kauffmann F Zur [illegible] Salmonella Sub Genera I II und III Acta path et

Kauffmann F & Rc [illegible] en Salmonella Diagnose Acta path et

FROM KAPTEIN W. WILHELMSEN OG FRUES BAKTERIOLOGISKE INSTITUTT, OSLO HEAD PROFESSOR S DICK HENRIKSEN, M.D

THE EFFECT OF THALIDOMIDE ON THE GROWTH CURVE OF A RIBOFLAVIN DEPENDENT MICROBE

By

TORE MIDTVEDT

Received 11 i 63

During the last year, numerous reports have appeared which indicate that the administration of thalidomide to women during early pregnancy may be followed by the birth of a malformed child A brief survey of the problems concerned has recently been given by *v Schrader & Sievers* (1962) In an attempt to explain the teratogenicity of thalidomide, a very interesting theory has been proposed by *Leck & Miller* (1962) Supported by clinical observations they point out that there are several similarities between the effect of thalidomide on human beings and those of riboflavin-deficiency in rats No animal experiments have as yet been published, however, which elucidate this problem

It has been the purpose of the present investigation to study the effect of thalidomide on the growth of riboflavin dependent microbes A brief report concerning some of the results have previously appeared in Norwegian (*Midtvedt* 1963 a, *Midtvedt* 1963 b)

MATERIALS AND METHODS

Bacterial Strains

As the ma n test m . .

Culture Technique

Growth curve analyses were performed in ro . . n . . dia-
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vita-

Species	Körper Antigene	Geissel Antigene	
		1 Phase	2 Phase
	Gruppe 58		
S basel	58	l z_{13} z_{28}	1 5
	Gruppe 59		
S betioky	59	k	(z)
	Gruppe 60		
S luton	60	z	e n x

ZUSAMMENFASSUNG

Es werden die Antigenformeln der bisher bekannten 131 *Salmonella species* des *sub genus* II in einer Tabelle zusammengestellt und mit den ca 700 *species* des *sub genus* I verglichen

Zu den O Gruppen A–E gehoren nur ca 30 % der *species* des *sub genus* II, aber ca 60 % derjenigen des *sub genus* I Beim *sub genus* II kommen also uberwiegend die hoheren O Gruppen vor, sodass dieses *sub-genus* auch in serologischer Hinsicht eine Zwischenstellung zwischen den *sub-genera* I und III (Arizona) einnimmt

Auch die H-Antigen-Verteilung innerhalb der 3 *sub genera* ist deutlich verschieden, sodass diese einen charakteristischen Antigenaufbau besitzen

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... ffmann White Scheme (VI) Acta path et

... r Salmonella Sub Genera I II und III ... Druck

... der serologischen Salmonella Diagnose ... 341 342 1962

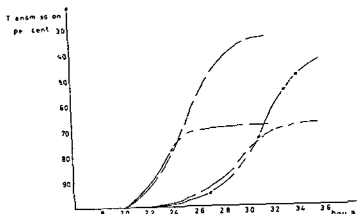


Fig 1

Rate of growth of Lactobacillus delbruckii in a medium containing varying concentrations of Riboflavine and with/without a concentration of Thalidomide of 100 mcg/ml

——— ——— ——— = Riboflavine 0 0030 mcg/ml Thalidomide 0 mcg ml
— — — — — — — = Riboflavine 0 0030 mcg/ml Thalidomide 100 mcg ml
——— + ——— + ——— = Riboflavine 0 0150 mcg ml Thalidomide 0 mcg/ml
——— ——— ——— — = Riboflavine 0 0150 mcg ml Thalidomide 100 mcg ml

Thal domide was suspended in ster le d st lled w ... [illegible]

[illegible] cent is destroyed by auto- ca ing (... de 1962)

Photometry

The growth rate was determined by turbidimetric readings in a Beckman Model C colorimeter with green filter This filter gives maximum transmittance at 524 mμ

Chemicals

Niacin and biotin were obtained commercially through Norsk Medisinaldepot riboflavin was obtained from E. Merck AG Darmstadt, Germany Thalidomide was kindly supplied by Norsk Astra A/S Oslo

RESULTS

The first problem which was attacked was the influence of thalido [illegible] requir Table 1 [illegible] have been recorded which demonstrate a significant change in the growth response of Lactobacillus delbruckii as the results of an addition of thalidomide The influence of thalidomide becomes apparent when the data are arranged in a growth diagram as shown in Fig 1 Apparently the lag phase is approximately 6 hours shorter when thalidomide is present than in the control without In contrast to this no significant change can be demonstrated in the other growth

TABLE 1

Rate of Growth of Lactobacillus delbrückii in a Medium Containing Varying Concentrations of Riboflavin Each Concentrations of Riboflavin Is Tested with and without a Constant Amount of Thalidomide

Riboflavin in mcg ml	Thalidomide in mcg ml	Inoculum in ml	Hours 18	20	21	23	24	25	26	27	28	29	30	31	32	33	34	36	41	48
0 0	0 0	0 0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
0 0	0 0	0 2	100	100	100							95		94	93	92	92	92	92	92
0 0	100	0 2	100	100	100	98	97	96	95	95	95	95			95					95
0 0025	0 0	0 2	100	98	98	98	97	97	96			88	84	78	76	75	75	75	71	71
0 0025	100	0 2	100	100	97	96	88	83	80	76	74	74	74	74	74	74	74		73	
0 0050	0 0	0 2	100	99	98	98	98	97	94			85	78	75	71	70	69	67	66	
0 0050	100	0 2	100	100	98	85	78	72	71	71	69	69	69	69	69	68	68	68	68	
0 0075	0 0	0 2	100	100	99	98		95	95	94	93	88	83	79	70	66	64	60	59	59
0 0075	100	0 2	100	99	99	81	75	67	62	61	60	59	59	59	59	59	59		58	
0 01	0 0	0 2	100	100	98	98	98	98	96	95		89	81	76	71	64	60	57	54	51
0 01	100	0 2	100	100	100	89	79	69	61	57	53	52	50	50	50	50	50		50	
0 015	0 0	0 2	100	100	100	99	99	97	95	95	93	87	81	74	62	56	50	44	36	35
0 015	100	0 2	100	100	100	88	79	69	58	48	41	40	36	36	35	35	35		35	
0 02	0 0	0 2	100	100	100	100	100	98	93	88	81	71		54		41	34	30	25	25
0 02	100	0 2	100	99	89	68	52	44	38	33	29	27	26	26	26	26	25		25	
0 03	0 0	0 2	100	100	100	98	98	97	94	89	83	75		56		45	36	31	18	17
0 03	100	0 2	100	98	91	67	53	45	39	34	28	24		22		19	19		18	

Each figure recorded is the mean of the reading of two different samples, and the results are given as per cent transmittancy

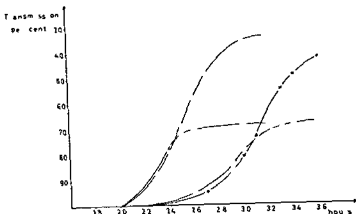


Fig 1

Rate of growth of Lactobacillus delbruckii in a medium containing varying concentrations of Riboflavine and with/without a concentration of Thalidomide of 100 mcg/ml

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——–——–—— = Riboflavine 0 0050 mcg/ml Thalidomide 100 mcg/ml
——+——+—— = Riboflavine 0 0150 mcg/ml Thalidomide 0 mcg/ml
—— —— —— – = Riboflavine 0 0150 mcg/ml Thalidomide 100 mcg/ml

Thal dom de —

that only a small part—about 5 per cent—is destroyed by autoclaving (*Fide* 1962)

Photometry

The growth rate was determined by turbidimetric readings in a Beckman Model C colorimeter with green filter This filter gives maximum transmittance at 524 mμ

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Niacin and biotin were obtained commercially through Norsk Medisinaldepot ri flavin was obtained from F Merck, AG Darmstadt Germany Thalidomide was kindly supplied by Norsk Astra A/S Oslo

RESULTS

The first problem which was attacked was the influence of thalidomide on the various phases of the growth curve of a riboflavin requiring microbe in which the growth was limited by riboflavin In Tabl some
ch in
of ar The influence of thalidomide becomes apparent when the data are arranged in a growth diagram as shown in Fig 1 Apparently the lag phase is approximately 6 hours shorter when thalidomide is present than in the control without In contrast to this, no significant change can be demonstrated in the other growth

TABLE 2

Rate of growth of Lactobacillus delbrückii in a Media Containing Constant Concentration of Riboflavin and Varying Concentration of Thalidomide

Riboflavin in mcg ml	Thalidomide in mcg ml	Inoculum in mcg ml	Hours 18	20	22	23	24	25	26	27	28	29	30	37
0.0	0.0	0.0	100	100	100	100	100	100	100	100	100	100	100	100
0.0	0.0	0.2	100	100			98		97			94		93
0.0	100	0.2	100	100			99		98			97		97
0.01	0.0	0.2	100	100	100	99	98	96	92	87	78	70	64	55
0.01	200	0.2	100	95	86	79	69	60	65	53	53	52	52	51
0.01	100	0.2	100	96	87	78	68	61	67	56	55	54	53	52
0.01	50	0.2	100	96	89	81	73	64	69	55	54	54	53	51
0.01	10	0.2	100	97	93	87	77	67	60	56	54	53	52	52
0.01	5	0.2	100	98	95	90	84	74	66	61	58	56	55	53
0.01	1	0.2	100	98	98	96	90	87	82	72	64	60	59	54
0.01	0.5	0.2	100	100	100	96	93	90	83	74	65	60	58	54
0.01	0.10	0.2	100	100	100	97	97	97	91	84	73	68	63	56
0.01	0.05	0.2	100	100	100	98	97	96	91	84	73	66	63	55

Each figure recorded is the mean of the reading of two different samples and the results are given as per cent transmittancy

TABLE 3

Rate of Growth of Lactobacillus arabinosus in Media Containing Varying Concentration of Niacin and Thalidomide

Niacin in mcg ml	Thalidomide in mcg ml	Inoculum in ml	Hours 15	16	17	18	19	20	21	22	23	24	25	26	27	29
0.0	0.0	0.0	100	100	100	100	100	100	100	100	100	100	100	100	100	100
0.0	0.0	0.2	100	100	100	100				94		90		88	88	88
0.0	100	0.2	100	100	100	100				96		91		89	88	88
0.02	0.0	0.2	100	93	96	90	83	74	58	45	34	28	25	23	21	20
0.02	100	0.2	100	99	95	88	81	70	53	40	31	26	23	22	21	19

Each figure recorded is the mean of the reading of two different samples and the results are given as per cent transmittancy

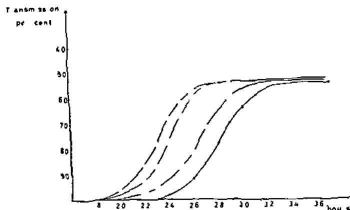


Fig 2

Rate of growth of Lactobacillus delbrückii in a media containing constant concentration of Riboflavin and varying concentrations of Thalidomide

—+—+—+— = Riboflavine 0 01 mcg/ml Thalidomide 0 mcg ml
—— —— —— = Riboflavine 0 01 mcg/ml Thalidomide 1 mcg ml
— — — — = Riboflavine 0 01 mcg/ml Thalidomide 10 mcg ml
— — — — — = Riboflavine 0 01 mcg ml Thalidomide 100 mcg/ml

TABLE 4

Rate of Growth of *Lactobacillus arabinosus in a Medium Containing Varying Concentrations of Biotin and Thalidomide*

Biotin in mcg ml	Thalidomide in mcg ml	Inoculum in mcg ml	Hours 16	19	23	24	25	29	35
0 0	0 0	0 2	100	100	100	100	100	100	100
0 0	0 0	0 0	100	100	98	96	90	90	90
0 0	100	0 2	100	100	97	95	89	89	89
0 00003	0 0	0 2	100	98	85	78	69	48	47
0 00003	100	0 2	100	99	84	77	66	46	45

Each figure recorded is the mean of the readings of two different samples and the results are given as per cent transmittance

phases Furthermore riboflavin is in each instance acting as the growth limiting factor

In a following series of experiments the quantity of thalidomide necessary in order to reduce the lag phase was studied From the data presented in Table 2 it may be seen that the shortening of the lag phase is indeed dependent on the concentration of thalidomide The shortest lag phase is found with a concentration of 100 mcg per ml Higher concentrations give no further changes in the pattern of the growth curve than that obtained with 100 mcg per ml When the concentration of thalidomide is reduced below 100 mcg per ml the lag phase is steadily increased At the concentration of 0 1 mcg per ml no effect can be observed on the lag phase which is then identical with that found in media

TABLE 2

Rate of growth of Lactobacillus delbrückii in a Media Containing Constant Concentration of Riboflavin and Varying Concentration of Thalidomide

Riboflavin in mcg/ml	Thalidomide in mcg/ml	Inoculum in mcg/ml	Hours											
			18	20	22	23	24	25	26	27	28	29	30	47
0.0	0.0	0.0	100	100	100	100	100	100	100	100	100	100	100	100
0.0	0.0	0.2	100	100			98		97			94		93
0.0	100	0.2	100	100			99		98			97		97
0.01	0.0	0.2	100	100	100	99	98	96	92	97	78	70	64	55
0.01	200	0.2	100	95	96	79	69	60	55	53	53	52	52	51
0.01	100	0.2	100	96	87	78	68	61	57	56	55	54	53	52
0.01	50	0.2	100	96	89	81	73	64	59	55	54	54	53	51
0.01	10	0.2	100	97	93	87	77	67	60	56	54	53	52	52
0.01	5	0.2	100	98	95	90	84	74	66	61	58	56	55	53
0.01	1	0.2	100	98	98	96	90	87	82	72	64	60	59	54
0.01	0.5	0.2	100	100	100	96	93	90	83	74	65	60	58	54
0.01	0.10	0.2	100	100	100	97	97	97	91	84	73	68	63	56
0.01	0.05	0.2	100	100	100	98	97	96	91	84	73	66	63	55

Each figure recorded is the mean of the reading of two different samples and the results are given as per cent transmittancy

TABLE 3

Rate of Growth of Lactobacillus arabinosus in Media Containing Varying Concentration of Niacin and Thalidomide

Niacin in mcg/ml	Thalidomide in mcg/ml	Inoculum in ml	Hours													
			15	16	17	18	19	20	21	22	23	24	25	26	27	29
0.0	0.0	0.0	100	100	100	100	100	100	100	100	100	100	100	100	100	100
0.0	0.0	0.2	100	100	100	100				94		90		88	88	88
0.0	100	0.2	100	100	100	100				96		91		89	88	88
0.02	0.0	0.2	100	99	96	90	83	74	58	45	34	28	25	23	21	20
0.02	100	0.2	100	99	95	88	81	70	53	40	31	26	23	22	21	19

Each figure recorded is the mean of the reading of two different samples and the results are given as per cent transmittancy

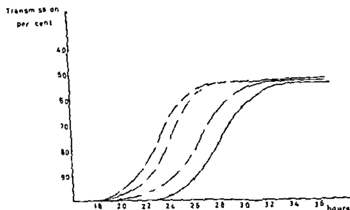


Fig 2

Rate of growth of Lactobacillus delbruckii in a media containing constant concentration of Riboflavin and varying concentrations of Thalidomide

—+——+ —+—— = Riboflavine 0 01 mcg/ml Thalidomide 0 mcg ml
— —— —— = Riboflavine 0 01 mcg/ml, Thalidomide 1 mcg ml
— —— —— = Riboflavine 0 01 mcg/ml, Thalidomide 10 mcg ml
— — — —— — = Riboflavine 0 01 mcg ml, Thalidomide 100 mcg/ml

TABLE 4

Rate of Growth of Lactobacillus arabinosus in a Medium Containing Varying Concentrations of Biotin and Thalidomide

Biotin in mcg ml	Thalidomide in mcg ml	Inoculum in mcg ml	Hours 16	19	23	24	25	29	35
0 0	0 0	0 2	100	100	100	100	100	100	100
0 0	0 0	0 0	100	100	98	96	90	90	90
0 0	100	0 2	100	100	97	95	89	89	89
0 00003	0 0	0 2	100	98	85	78	69	48	47
0 00003	100	0 2	100	99	84	77	66	46	45

Each figure recorded is the mean of the reading of two different samples and the results are given as per cent transmittancy

phases Furthermore, riboflavin is in each instance acting as the growth limiting factor

In a following series of experiments, the quantity of thalidomide necessary in order to reduce the lag phase was studied From the data presented in Table 2 it may be seen that the shortening of the lag phase is *indeed dependent* on the concentration of thalidomide The shortest lag phase is found with a concentration of 100 *mcg per ml* Higher concentrations give *no* further changes in the pattern of the growth curve than that obtained with 100 *mcg per ml* When the concentration of thalidomide is reduced below 100 *mcg per ml*, the *lag* phase is steadily increased At the concentration of 0 1 *mcg per ml*, no effect can be observed on the lag phase which is then identical with that found in media

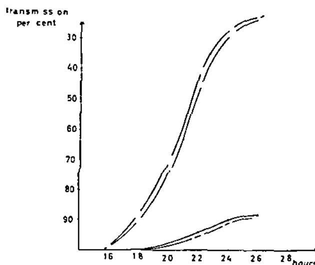


Fig 3

Rate of growth of Lactobacillus arabinosus in a medium containing varying concentrations of Niacin and Thalidomide

——+——+——+—— = Niacin 0 mcg/ml, Thalidomide 0 mcg/ml
—— — —— — —— = Niacin 0 mcg/ml, Thalidomide 100 mcg/ml
—— ——·——· = *Niacin 0 02 mcg/ml, Thalidomide 100 mcg/ml*
—— —— ——·— = Niacin 0 02 mcg/ml, Thalidomide 0 mcg/ml

without thalidomide This thalidomide effect has also been illustrated by the graphs presented in Fig 2

The *next problem to be studied* was *that of the specificity of* this thalidomide effect Growth experiments in similar technique were performed with the vitamins niacin and biotin as recorded in Table 3 and Table 4 From the data presented no significant effect from thalidomide can be demonstrated on the growth curves in microbes in which *the growth is limited by either niacin or biotin The thalidomide* insensitive niacin assays have been illustrated in the graphs of Figure 3

DISCUSSION

When the riboflavin dependent lactobacillus delbruckii is growing with riboflavin as the limiting factor in the presence of thalidomide, the lag phase is significantly shortened This decrease in time is independent of the concentration of riboflavin, and the final production of bacteria is still determined—limited—by the riboflavin available The total synthesis of bacteria is accomplished approximately 6 hours earlier when thalidomide is present than when absent

A concentration of 100 mcg per ml of thalidomide has a maximum influence on the lag phase under the conditions of the assay Higher concentrations do not further change the growth curves In this connection it should be remarked that the concentration of 100 mcg per ml corresponds to the maximal amount of thalidomide actually dissolved under these conditions

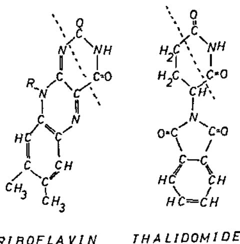


Fig 4

Formulae of riboflavin and thalidomide The CO NH CO sequence is shown at the top of each formula

Experiments with other vitamins as growth limiting factors may be taken to indicate a specificity in the relation between thalidomide and riboflavin even if too definite conclusions would be premature at the present stage

The interpretation of these findings is as yet far from clear A comparison of riboflavin and thalidomide, such as drawn in Fig 4, indicate that both have the sequence—CO NH CO— It has been assumed (*Leck & Miller* 1962) that the NH group is one point at which the coenzymes which contain riboflavin combine with their apo-enzymes Thus, it seems conceivable that thalidomide excerts its toxic effects, in the embryo as well as in adult, either by competing with riboflavin-containing coenzymes for attachment to their apoenzymes or by inactivation of the vitamin by combining with it In either case, the effect would be that of a vitamin deficiency In the biological systems used in the present investigation, such a deficiency should have resulted in signs which indicate a retardation of the growth, *i e* lengthening of the lag phase, a flat logarithmic phase, and probably a decrease in the amount of cells synthetized Instead of this, we observed an acceleration in the start of growth when thalidomide is added

It might well be that thalidomide, on account of its similarities with

In any case, the results presented indicate new possibilities for the study of the effect of thalidomide on the metabolism, since many well known microbial systems are at hand Such systems include the elaborate instruments of microbial genetics

SUMMARY

The effect of thalidomide on the growth of some vitamin requiring microbes has been examined

No effect on the growth curve is found when niacin or biotin is used as the growth limiting factor

When riboflavin is the limiting factor, thalidomide changes the growth curves This effect is concerned with the lag phase which is significantly shortened The effect is dependent of the concentration of thalidomide, and independent of the concentration of riboflavin

The experiments demonstrate a new, reproducible biological effect of thalidomide in addition to the known sedative effect and the probable teratogenic effect in man

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1 *Eide L* Personal communication 1962
2 *Leck I M & Miller E L M* Incidence of malformations since the introduction of thalidomide British Medical Journal 2 16 1962
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4 *Midtvedt T* Effekten av tahlidomide på veksten av en riboflavinavhengig mikrobe Tidsskrift for den Norske Lægeforening 1963 (In press)
5 *v Schrader & Sievers* Thalidomide und Missbildungen Therapeutische Briefe Grunenthal 8 33 1962

TUBERCULIN PRODUCTION

I. Yield of Tuberculoprotein from Various Media

By

MOGENS MAGNUSSON, HYUN KYU KIM and
M. WEIS BENTZON

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The methods used in the sundry laboratories for preparing tuberculin (*Seibert* 1934, *Seibert & Glenn* 1941, *Jensen* 1938, *Lind* 1947, 1948, *McIntosh & Konst* 1947, *Bretey & Lamensans* 1951, *Lamensans, Grabar & Bretey* 1951, *Green* 1953, *Svenkerud* 1955, *Magnusson & Bentzon* 1958, *Desbordes* 1958, *Ministry Hlth. Welf., Jap.* 1961) vary in many respects, there are differences in the media and strains, the incubation time for the cultures, the method of concentration, and the purification procedure, etc. It is also obvious from the literature that the yield of tuberculin varies and possibly there are differences in the specificity of the tuberculin produced by the various methods. However, it is not possible to attribute the variation in the yield to definite factors in the production method since so many aspects vary simultaneously. In order, therefore, to obtain a better basis for selection of a rational production method, a series of experiments were carried out to study the variations in tuberculin yield in relation to the composition of the medium, the strain, and the incubation period. The experiments also included comparison between the tuberculin content of culture filtrates and the amount of tuberculin which can be extracted from the bacterial cells. The results of these experiments will be published in a series of articles.[1]

In the present work, comparison is made between seven different media, four of which are those commonly employed, viz. Sauton (*Sauton* 1912), Long (*Long & Seibert* 1926), Dorset as modified by the Bureau of Animal Industry, USA (*Dorset* 1934), and glycerol broth. Three others were included because previous experiments with them had shown high yields of tuberculoprotein, viz. Lind bII medium (*Lind* 1948), glucose medium, and P medium. Three strains of *Mycobacterium tuberculosis* were used for the comparison, and the incubation period

[1] The experimental work was performed in 1955.

SUMMARY

The effect of thalidomide on the growth of some vitamin requiring microbes has been examined

No effect on the growth curve is found when niacin or biotin is used as the growth limiting factor

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Synthetic media The composition of the synthetic media is shown in Table 1 The media were prepared by dissolving the salts in a small quantity of the water, if necessary by heating After adding the rest of the water and the glycerol the pH was adjusted in the Sauton and glucose media by adding concentrated NH_3 Distribution was made of 180 ml amounts into small flasks which were sterilized by autoclaving (20 minutes at 120° C) In the case of the glucose medium, the glucose was sterilized separately as an 18 per cent (w/v) solution and added to the medium aseptically [2]

The Lind bII medium was turbid when prepared and a small precipitate was found in each flask after sterilization, this disappeared during growth of the cultures A small constant precipitate formed during autoclaving of the Dorset medium

Strains

Three virulent strains of *Mycobacterium tuberculosis* were used One strain T3487 was freshly isolated from a patient at the time of the experiment The two others E9656 and U1921 were old laboratory strains isolated from patients more than 10 years previously

Two cultures of each strain grown on 180 ml Sauton medium were used for ere 16 days old by the medium The membrane with a .. and a thin, regular membrane covered the surface of the medium

Inoculation and Incubation

A pellicle with a diameter of about 10 mm was used as inoculum for each flask. The cultures were incubated at 38° C and one or two flasks of each medium were sterilized by heating in streaming steam for one hour after 8 days 2 3 4 5 6 7, 8

TABLE 1
Composition of Synthetic Media

		Sauton	Long	Dorset	Lind b II	Glucose	P medium
Asparagine	g	4	5	14	8	6	12
Citric acid	g	2			0.4*	2	2
Potassium ortho phosphate di H	g	0.5	3	1.8	2.22	1.5	2
Magnesium sulphate	g	0.5	1	1.5	0.5	0.25	0.5
Ferric ammonium citrate	g	0.05	0.05		0.05	0.05	0.05
Sodium chloride	g		§				
Sodium carbonate	g		2				
Sodium citrate	g			0.9			
Ferric citrate	g			0.3			
Ammonium citrate	g		5				
Glycerol	ml	50	40	100†	60	30	60
Glucose	g			10		10	
Water distilled	ml	1 000			950**		
Water tap	ml		1 000	1 000°		1 000	950
Ammonium hydroxide conc	ml	2.2				1.8	
pH before autoclaving		7.4	7.2	6.9	6.7	6.4	6.8
pH after autoclaving		7.0	6.8	6.8	6.7	6.4	7.0

* Original 0 2 g § Original 2 0 g † Original 100 g
° Original distilled water ** Original tap water

[2] If the glucose is heated (sterilized) together with the phosphate toxic compounds are formed (Wong 1937 *Dubos & Middlebrook* 1947)

varied from one to nine weeks The protein content of the culture filtrates was measured and is considered in relation to the growth of the bacteria and the pH of the culture filtrates

The literature contains many reports concerning the yield of tuberculoprotein and tuberculin by cultivation of tubercle bacilli on media of varying composition

Wong & Weinzirl (1936) modified Long medium by replacing asparagine with ammonium malate and adding glucose They obtained the same amount of tuberculin with the ammonium malate medium as with the asparagine medium, but in approximately half the time Later *Wong* (1937) described another synthetic medium, a glucose-sucrose medium, which gave three times as much protein as could be obtained on Long medium at the end of six weeks *Henley & Le Duc* (1939) found that the yield of protein increased when the amount of asparagine and/or glycerol was increased from 4 and 20 g per litre respectively in a synthetic medium which contained the other compounds necessary for the growth *Lind* (1948) measured the yield and activity of tuberculin prepared on modifications of Sauton medium, varying the concentration of glycerol and asparagine, and ammonium ferric citrate The yield of purified tuberculin increased as the asparagine concentration increased, but the activity was reduced simultaneously Lind modified the medium further by increasing the phosphate concentration (Lind bII medium) and obtained high yield but low activity of the PPD *Asami et al* (1953 a, b) measured the protein yield obtained on Sauton and Dorset media and found a higher yield on the latter *Svenkerud* (1955) found that the yield of protein increased if sodium hydroxide and acetic acid were added to the Sauton medium However, the amount necessary to obtain maximum protein yield varied according to the strain used *Gottshall* (1962) found that the dry weight of organisms was greater in cultures of tubercle bacilli grown in Henley's synthetic medium (*Henley* 1929) to which zinc and copper were added and supplemented with calcium, than in those without calcium The quantity of tuberculoprotein in the culture filtrate was lower in cultures to which calcium had been added than in cultures without calcium

MATERIALS AND METHODS

Media

Glycerol broth The ingredients were —500 g minced veal 10 g Wittes peptone
... 1000 ml tap water
... rigerator overnight with half the quantity
the fluid was pressed out of the meat and
e other half of the water The liquids were
ed and the medium heated and boiled for
5 minutes Alkalization was ca... with 5 N sodium hydroxide so that after boiling for a further 5 minutes the pH was 7 6 to 7 7 The medium was filtered through filter paper and measured up to volume and finally the glycerol was added

The medium was measured out in 180 ml volumes into small flat bottomed flasks and sterilized by autoclaving for 20 minutes at 120° C The pH after autoclaving was 7 2

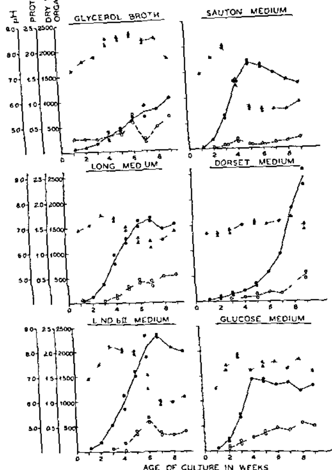


Fig 1

Dry weight of organism (●), pH (Δ) and protein content (○) of culture filtrate of *Mycobacterium tuberculosis* (Strain T3487) cultured on various media in relation to age of culture

The growth rate for the two strains was the same on *Lind bII medium* as on Sauton medium, but the total bacterial crop was higher on the former (about 2300 and 2700 mg) Strain U1921 shows quite strong autolysis on Lind bII medium

While T3487 multiplied with the same speed and almost the same maximum yield of organism on *glucose medium* as on Sauton and Long media, the growth of U1921 was more rapid on *glucose medium* com-

and 9 weeks After sterilization, the cultures were stored in the refrigerator until time of analysis (1 day to 3 weeks)

Analysis

Dry weight The content of each flask was poured on to a piece of filter paper (weight determined previously) placed in a Buchner funnel and the culture filtrate removed by means of suction through the paper The filter paper containing the cells was placed in open metal racks and dried at about 105° C for 24 hours before weighing

pH The pH of the culture filtrates was determined electrometrically on a sample of the filtrate, using a Radiometer pH Meter 22 with glass electrode

Precipitation and estimation of protein A sample of 10 ml from each filtrate was precipitated with 1 1 ml of 50 per cent trichloroacetic acid After standing in the refrigerator for not less than 24 hours, the precipitates were centrifuged washed twice with 10 ml of 2 per cent trichloroacetic acid and once with water Finally the precipitates were dissolved in 1 or 2 ml of 1/10 N sodium hydroxide and the protein content determined by the biuret reaction, using the Weichselbaum biuret reagent (*Weichselbaum* 1946) The readings were made in a Beckmann spectrophotometer at wave length 540 mμ using cuvettes containing 0 3 ml (light path 1 cm) The protein content (mg/ml) was calculated from the Beckmann reading by multiplication by 0 9 this factor being determined from Kjeldahl analysis of a reference preparation of tuberculoprotein included in the biuret analysis

RESULTS

The results of the experiment are shown in Figs 1 to 3

Growth of Tubercle Bacilli on the Various Media

Generally the dry weight of organism increased during the period from 2 to 5–7 weeks Later autolysis of the bacilli could be seen as the weight of the culture decreased In a few cases, however, multiplication was so slow that the weight of the cultures increased throughout the whole observation period

T3487 and U1921 (Figs 1 and 2) On *glycerol broth* the growth rate of T3487 and U1921 was slower and the total bacterial crop—about 1000 mg—smaller than on the synthetic media On *Sauton medium* the total bacterial crop was about 1700 mg for T3487 and about 2000 mg for U1921, in both cases with a culture period of five weeks After nine weeks the weight of the culture had decreased to about 1300 mg and 1500 mg respectively because of autolysis

On *Long medium* the growth of the two strains was much the same as on Sauton medium, both as regards yield of bacilli and speed of growth Autolysis of the bacilli was not so pronounced

Growth of the two strains on *Dorset medium* was slow However, the total weight of the bacterial cells after 8 to 9 weeks growth was large—about 2500 and 2700 mg for the two strains—and it is even possible that these values do not express the maximum yield of bacilli on the medium [3] The differences between the two flasks of seven- and eight-weeks-old cultures of U1921 were large

3 There was no observation after 9 weeks culture of U1921 because of contamination

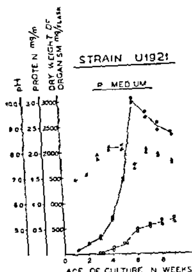


Fig 2 a

Dry weight of organism (●) pH (Δ) and protein content (○) of culture filtrate of *Mycobacterium tuberculosis* (Strain U1921) cultured on a synthetic medium in relation to age of culture

cially in Long Lind bII and glucose media,[4] where the variations between cultures of the same age, and particularly the variations from week to week, were larger than the corresponding variations for the two other strains Such irregularities in the growth have occurred also in some later experiments with other strains, and in these cases also on Sauton medium In drawing the figure it has been presumed that there are two different forms of growth, a and b The one (a) is characterized by large total growth and rapid acidification of the culture filtrate, possibly followed by autolysis The other (b) is characterized by slower growth, smaller total bacterial crop, and lesser change in pH probably due to sinking of the pellicle at a different period before sterilization of the culture (see under Discussion) Growth of this strain on the broth was also peculiar There were no signs of growth from the second to the fifth week but later slow multiplication was again evident

Despite this divergence, multiplication of E9656 on the various media shows some of the same characteristics as the other strains *The total* bacterial crop was small on the broth (800 mg), not much different in Sauton, Long and glucose media (1700, 2000 and 1800 *mg*), *and largest in* Lind bII medium (2400 mg)

However, in contrast to the results with the other two strains, the total crop of bacteria on Dorset medium was relatively small (1500 mg)

[4] Due to a technical mishap it was not possible to record some of the points on the growth curve for Sauton medium

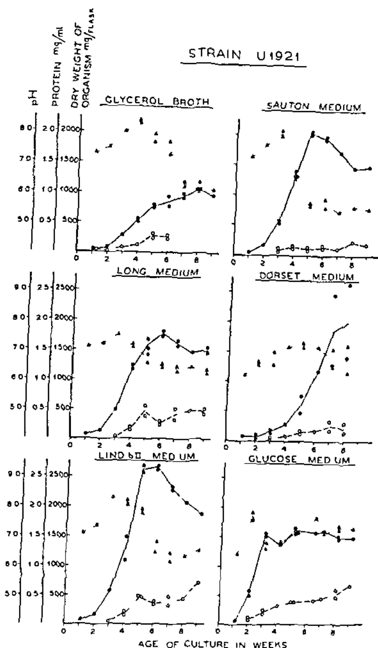


Fig 2

Dry weight of organism (●) pH (△) and protein content (○) of culture filtrate of *Mycobacterium tuberculosis* (Strain U1921) cultured on various media in relation to age of culture

pared with the other media, the maximum weight being reached after four weeks Autolysis of the bacilli on glucose medium was not pronounced

Only strain U1921 was cultivated on the *P-medium* (Fig 2 a) The total bacterial crop (3000 mg) was higher in this medium than in any of the others, and autolysis was fairly strong

Strain E9656 The growth of this strain (Fig 3) was irregular, espe-

concentration with the age of the culture, while others show a peak On Lind bII medium the protein curve for strain E9656 (Fig 3) shows irregularity similar to that for the growth curve

The protein curve for T3487 on *broth medium* (Fig 1) shows a fairly large protein content after one and two weeks The corresponding bacterial crops were very small Thus, it would seem probable that the protein found was not produced by the tubercle bacilli but probably originated from the broth The results of protein analysis on the broth medium are therefore not comparable with those on the synthetic media (which do not contain protein) and will not be considered further

In *Sauton medium* (Figs 1, 2, 3) the protein content increased very slightly with the age of the culture However, the largest protein contents (0 2 to 0 3 mg ml) were small compared with those of the other synthetic media

In *Long medium* also (Figs 1, 2, 3) the protein content increased the longer the culture period but here the maximum protein contents observed were larger (0 6 to 1 4 mg ml)

The maximum protein contents obtained on *Dorset medium* (Figs 1, 2 3) were slightly smaller than those found on Long medium (0 3–1 1 mg/ml)

On *Lind bII medium* (Figs 1, 2 3) a high protein content was obtained for all three strains (0 7–1 6 mg ml)

On the *glucose medium* (Figs 1, 2, 3), the protein content increased regularly with the age of the culture, and the maximum content was fairly high for all three strains (0 6–0 8 mg ml)

On the *P medium* (Fig 2 a), the highest protein concentration measured was 0 7 mg ml

Measurement of pH

The hydrogen ion concentration was measured after heat sterilization of the cultures, and the pH was found to vary between 5 4 and 8 7 However, the changes in pH evoked by growth of the tubercle bacilli differed from one medium to another In Sauton and Lind bII media, the pH first increased to 8 0 or more, later decreased to about 5 5 to 6 0, and sometimes increased again during the latter part of the observation period In the other media the variations in pH were smaller

As regards strain F9656 (Fig 3), the large variations in growth and protein content for Lind bII medium were also reflected by the pH measurements The cultures with large amounts of growth were more acid and had lower protein content than those containing a smaller number of bacteria

DISCUSSION

It is a general assumption that tuberculoprotein and tuberculin are released into the culture filtrate of tubercle bacilli by autolysis of the cells (*Corper & Cohn* 1943, 1944, *Green* 1953 *Svenkerud* 1955 *Paterson*

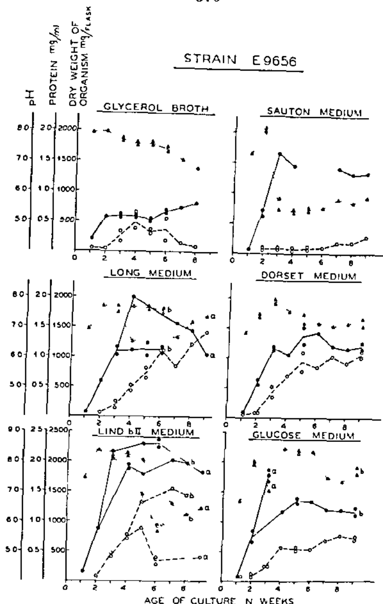


Fig. 2

Dry weight of organism (●), pH (△) and protein content (○) of culture filtrate of *Mycobacterium tuberculosis* (Strain E9656) cultured on various media in relation to age of culture
For explanation of a and b curves see text

As compared with T3487 and U1921, the growth rate of E9656 was high, and in particular multiplication of E9656 was not delayed in Dorset medium as it was with the two other strains. Autolysis in the cultures of E9656 could not be estimated with certainty due to the irregular growth.

Amount of Protein Present in the Culture Filtrates

The curves for the protein content of the culture filtrates show different patterns. Some of them indicate a regular increase of the protein

concentration with the age of the culture, while others show a peak On Lind bII medium the protein curve for strain L9656 (Fig 3) shows irregularity similar to that for the growth curve

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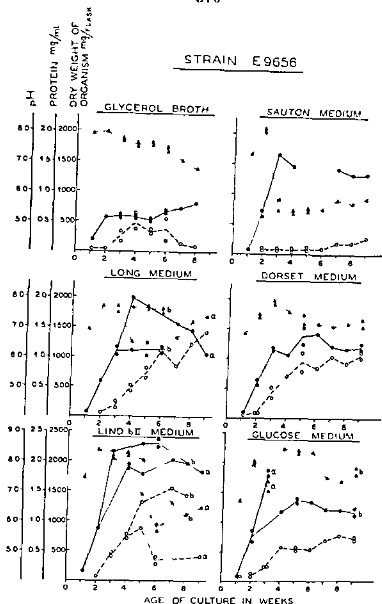


Fig 3

Dry weight of organism (●), pH (△) and protein content (○) of culture filtrate of *Mycobacterium tuberculosis* (Strain F9656) cultured on various media in relation to age of culture

For explanation of a and b curves see text

As compared with T3487 and U1921, the growth rate of E9656 was high, and in particular multiplication of E9656 was not delayed in Dorset medium as it was with the two other strains Autolysis in the cultures of E9656 could not be estimated with certainty due to the irregular growth

Amount of Protein Present in the Culture Filtrates

The curves for the protein content of the culture filtrates show different patterns Some of them indicate a regular increase of the protein

concentration with the age of the culture, while others show a peak On Lind bII medium the protein curve for strain E9656 (Fig 3) shows irregularity similar to that for the growth curve

The protein curve for T3487 on *broth medium* (Fig 1) shows a fairly large protein content after one and two weeks The corresponding bacterial crops were very small Thus, it would seem probable that the protein found was not produced by the tubercle bacilli but probably originated from the broth The results of protein analysis on the broth medium are therefore not comparable with those on the synthetic media (which do not contain protein) and will not be considered further

In *Sauton medium* (Figs 1, 2, 3) the protein content increased very slightly with the age of the culture However, the largest protein contents (0 2 to 0 3 mg/ml) were small compared with those of the other synthetic media

In *Long medium* also (Figs 1, 2 3) the protein content increased the longer the culture period, but here the maximum protein contents observed were larger (0 6 to 1 4 mg/ml)

The maximum protein contents obtained on *Dorset medium* (Figs 1, 2, 3) were slightly smaller than those found on Long medium (0 3–1 1 mg/ml)

On *Lind bII medium* (Figs 1, 2, 3) a high protein content was obtained for all three strains (0 7–1 6 mg/ml)

On the *glucose medium* (Figs 1, 2, 3), the protein content increased regularly with the age of the culture, and the maximum content was fairly high for all three strains (0 6–0 8 mg/ml)

On the *P-medium* (Fig 2 a), the highest protein concentration measured was 0 7 mg/ml

Measurement of pH

The hydrogen ion concentration was measured after heat sterilization of the cultures, and the pH was found to vary between 5 4 and 8 7 However, the changes in pH evoked by growth of the tubercle bacilli differed from one medium to another In Sauton and Lind bII media, the pH first increased to 8 0 or more, later decreased to about 5 5 to 6 0, and sometimes increased again during the latter part of the observation period In the other media the variations in pH were smaller

As regards strain E9656 (Fig 3), the large variations in growth and protein content for Lind bII medium were also reflected by the pH measurements The cultures with large amounts of growth were more acid and had lower protein content than those containing a smaller number of bacteria

DISCUSSION

It is a general assumption that tuberculoprotein and tuberculin are released into the culture filtrate of tubercle bacilli by autolysis of the cells (*Corper & Cohn* 1943, 1944, *Green* 1953, *Svenkerud* 1955, *Paterson*

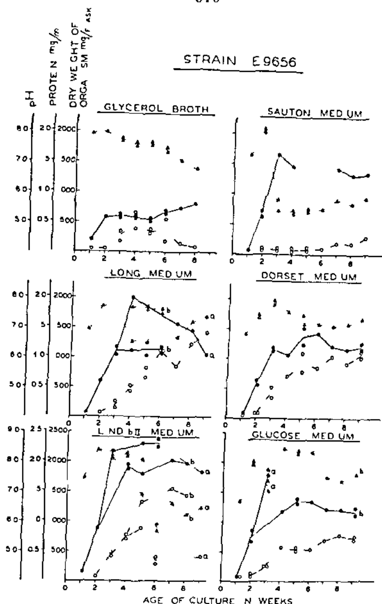


Fig 3

Dry weight of organism (●) pH (△) and pr tein c ntent (○) f culture filtrate of Mycobacterium tuberculosis (Strain F9656) culture l (n varieu media in relation to age of culture
F r explanation of a an l b curves see text

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Amount of Protein Present in the Culture Filtrates

The curves for the protein content of the culture filtr ites show dif ferent p itterns Some of them indicate a regul ir incre ise of the protein

strains U1921 and T3487 on Sauton medium Lind's results (1948) indicate a relationship between tuberculin yield and pH of the culture filtrate as regards *some* strains of *M tuberculosis* In addition to the variations in pH, therefore, there would seem to be other factors which exert an influence on the content of tuberculoprotein in the culture filtrate and the tuberculin yield The observations of *Baisden* (1951), *Paterson et al* (1958), *Dekker & Huitema* (1958) and *Gottshall* (1962) show that zinc, copper, calcium and other trace elements may be deficient in some lots of synthetic media used in tuberculin production, causing the pellicle to sink before it is fully grown, and resulting in a smaller total bacterial crop and a varying yield of tuberculoprotein Sinking of the pellicle of various strains of *M tuberculosis* and the BCG strain of *M bovis* can be prevented by adding zinc and other trace elements to deficient lots of synthetic media (*Williston et al* 1958, 1962, *Pouli Pateraki et al* 1962) Unfortunately, in the present study it was not observed whether the pellicles had sunk in some of the flasks before heat sterilization of the cultures However, this was observed in a subsequent study with the BCG strain and this is believed to be the explanation of the irregular growth of strain E9656 on some media in the present study It has been observed previously by others (*Paterson et al* 1958, *Dekker & Huitema* 1958), that various strains of *M tuberculosis* show a varying tendency to sink, this is probably the explanation for the regular growth of the two other strains on the same lots of media in the present study

In the present study there is *autolysis* of the bacteria as regards strain U1921 (Fig 2) when cultured for more than five to six weeks on Sauton, Lind bII and P media, and simultaneously a certain increase in the protein content of the culture filtrates (Fig 2) However, the formation of protein is just as rapid, or even more rapid, in the period from 3 5 weeks on Lind bII medium and from 3–6 weeks on P medium, where autolysis is not evident It would appear, therefore, that at least part of the tuberculoprotein, especially in the filtrates of the younger cultures, had been formed not because of autolysis of the cells, but by some other process

In the present work, only the tuberculoprotein content of the culture filtrates has been considered A subsequent paper will report the results of extraction experiments with the bacteria and measurement of the tuberculin activity of some of the culture filtrates and extracts

SUMMARY

Glycerol broth and some synthetic media, including Sauton, Long and Dorset media, were inoculated with three virulent strains of *Mycobacterium tuberculosis* After incubation at 38° C for one to nine weeks, the weight of culture, pH and protein content of the culture filtrate were determined for the heat-sterilized cultures

et al 1958), but direct experimental proof of this has not been demonstrated and the actual mechanism of their formation remains obscure (*Takehara* 1957) Frequently culture filtrates with pH 4–5 have lower protein content than filtrates with pH 7–8 (*Wong* 1937, *McIntosh & Konst* 1943, 1949, *Svenkerud* 1955, *Paterson et al* 1958, *Magnusson & Bentzon* 1958, *Asami et al* 1959a) In contrast to observations by *Szucs* 1955, *McIntosh & Konst* 1949 and *Svenkerud* 1955 found no relationship between the total bacterial crop of different strains of *M tuberculosis* and the yield of tuberculoprotein obtained on synthetic medium

In the *present experiment, the protein content of the culture filtrate* increased regularly with the growth of the bacteria during three to four weeks At that time the largest yields of tuberculoprotein were found in the glucose medium, probably due to the rapid growth of the tubercle bacilli in this medium For older cultures, either Lind bII, Long or glucose medium showed the largest content of tuberculoprotein, dependent on the strain and age of culture On Sauton medium the yield of tuberculoprotein was small, irrespective of age of culture

Considering all the media as a whole, the total crops of bacteria were somewhat larger with U1921 than with E9656 However, the protein yield obtained with E9656 was on an average nearly twice as large as that obtained with U1921 For all three strains, about the same total crops of bacteria were obtained on Sauton, Long and glucose media, *but the protein yield on Sauton medium was less than half of that in* the two other media Thus, in the present work, as in the reports of *McIntosh & Konst* 1949, and *Svenkerud* 1955, there is no relationship between the total bacterial crop of bacteria and the protein content of the culture filtrate

The pH variations contribute to the varying protein content of some of the culture filtrates from the fully-grown cultures in the present experiment This is evident in Fig 1, which shows that the protein yield with strain T3487 on Sauton, Long, Lind bII and glucose media decreases and increases according to the changes in pH, with incubation periods longer than four to six weeks The same correlation probably also exists when E9656 is cultured on Sauton medium (Fig 3)

Furthermore, the small yields of tuberculoprotein with the fully-grown cultures of all three strains on Sauton medium are probably also due to the low terminal pH values This has been shown more directly in subsequent experiments by varying the pH in fully-grown cultures of tubercle bacilli on Sauton medium immediately prior to sterilization, and by measuring the yields of tuberculoprotein obtained in the culture filtrates

However, as regards strain U1921 cultured on Long and Lind bII media (Fig 2), the correlation between pH and the protein content of the culture filtrates is not evident

Magnusson & Bentzon (1958) found a definite relationship between pH and tuberculin yield for strain E9656 and less dependence for

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The crops of bacteria obtained were larger on the synthetic media than on glycerol broth. Within the synthetic media group, differences were found both in total bacterial crop and in growth rate of the bacteria, the largest crops being obtained in media with a high content of asparagine and potassium dihydrogen phosphate (Lind bII and P medium).

As regards the yield of tuberculoprotein in the culture filtrates, none of the synthetic media was consistently better than the others, the results of the comparison being dependent on the strain and the age of the culture. However, the yield of tuberculoprotein on Sauton medium was always the lowest.

No simple relationship was found in the fully grown cultures between the total crop of bacteria and the protein content of the culture filtrates. The protein yield for one of the strains could be twice as large as that of another, although the total bacterial crop was not larger.

The culture filtrates with pH 5.6 had a lower protein content than those with a higher final pH, and the low protein yield on Sauton medium was due to the low final pH in this medium.

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IV 7 ml subc 1 ml i d and 2 ml i v given on the same day no booster

The animals in all 8 groups were bled by heart puncture 1 week after the last injection The sera were inactivated at 56° C for 30 min and stored at —20° C

Incubation Temperature

A temperature of 34° C was used for growth and incubation of the LSc 2ab strain while 37° C was employed for the Mahoney strain

Serum Titrations

0 25 ml of 2 fold serum dilutions were mixed with 0 25 ml virus dilution containing 160–200 TCID 0 per 0 2 ml After incubation for one hour 0 2 ml amounts of each mixture were inoculated into two MK roller tubes The tubes were rotated for at least 4 days and were read on the 4 and 7 day The serum titres were calculated from the 7 day s reading using *Karber* s method

Plaque Technique

Monolayers of MK cells were grown in Brockway bottles These bottles have a flat side with an area of 32 0 sq cm

The bottles were inoculated with 2 3 × 10^6 versenated cells in 20 ml of Hanks' BSS with 0 5 per cent lactalbumine hydrolysate and 2 per cent calf's serum The cell layers were used for inoculation when confluent i e on the 3 or 4 day

After washing with Earle s BSS the cell layer were inoculated with 0 2 ml of virus dilution containing 10^2–$10^{2\cdot5}$ PFU (plaque forming units) and were subsequently incubated for 1 hr at 34° or 37° C

The agar overlay then added consisted of

A 2 2 per cent Noble s agar melted and kept at 43° C

B 2 × Earle s BSS containing 1 per cent lactalbumine hydrolysate 0 2 per cent yeast extract 0 2 per cent bovine albumin and 0 005 per cent neutral red preheated to 37° C in a water bath

A and B were mixed in equal portions and poured into the bottles 10 ml per bottle the thickness of the overlay being 3 mm The compos i on of the [illegible]

[illegible] and [illegible] ml of a

[illegible] numbers and mean diameters were read on each of the subsequent 7 days

RESULTS

The antisera were titrated simultaneously in one experiment The logarithms on the base of 2 of these titres are recorded in Table 1 Some general trends may be seen from this table

a) The serum titres are found to increase with the length of the immunization period but they are not correlated to the number of injections nor to the total amount of virus employed in the present experiments i e 4 ml containing $10^{6\cdot9}$ $TCID_{50}$ per 0 2 ml (= $10^{8\cdot2}$ $TCID_{50}$ LSc,2ab virus when using method II) and 10 ml containing $10^{\cdot4}$ $TCID_{50}$ per 0 2 ml (= $10^{9\cdot1}$ $TCID_{50}$ Mahoney virus when using method I and IV)

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SERODIFFERENTIATION BETWEEN TYPE 1 POLIOVIRUSES WITH ANTISERA PRODUCED IN RABBITS USING FOUR DIFFERENT IMMUNIZATION SCHEMES

By

KNUD SIBONI and HERDIS VON MAGNUS

Received 23 ii 63

Work on the epidemiology of poliomyelitis and especially the need for differentiation between wild and attenuated vaccine strains has brought forward various methods for intratypic serological differentiation *McBride* (4) used neutralization kinetics based on the work by *Dulbecco et al* (1) *Wecker* (7) and *Nakano & Gelfand* (5) employed plaque neutralization, and *Wenner* (8) and *Gard* (2) found end-points from roller tube neutralization tests usable

The present work was done with the purpose of finding a practical and preferably quick method for production of rabbit antisera, which would prove useful for serodifferentiation of various type 1 poliovirus strains

MATERIALS AND METHODS

Strains of Virus

a) Mahoney Y-1871 2 monkey kidney (MK) tissue culture passage
b) LSc 2ab Y-4116 1' MK tissue culture passage
Both strains were kindly supplied by Dr *Albert B Sabin*

Stock Viruses

MK tissue cultures in Roux bottles were grown in Hanks BSS (basic salt solution) with 0 5 per cent lactalbumine hydrolysate and 2 per cent calf s serum They were changed into medium 199 before use After complete degeneration of the cells the tissue culture fluid was harvested and frozen at 20° C in 1 ml portions The two suspensions were labelled S 9 Mahoney and S 10 Lsc 2ab They had titres of $10^{7.4}$ $TCID_{50}$ per 0 2 ml and $10^{6.9}$ $TCID_{50}$ per 0 2 ml respectively

Antisera

Groups of 6 rabbits weighing 3000-3500 g were immunized with one of the two type 1 viruses in 4 different ways

I 9 ml subcutaneously (subc) and 1 ml intradermally (i d) and 12 weeks later 1 ml intravenously (i v) This method has been used by *Sabin* (6)

II 1 ml i v weekly for 3 weeks and 4 weeks later 1 ml i v

III 0 5 ml i v every 2 day during 2 weeks and 1 week later 1 ml intraperitoneally This method has been used by *McBride* (4)

b) Sera from rabbits immunized with the LSc,2ab strain show a higher titre against the homologous strain as compared to the heterologous (Mahoney) strain. However, the Mahoney strain is neutralized by LSc sera nearly to the same extent as by Mahoney antisera.
Sera from rabbits immunized with the Mahoney strain have about the same titre against the homologous and heterologous virus strain. From this rule Mahoney antisera 13 and 102 are striking exceptions as they show lower titres against the heterologous strain.

c) Sera from rabbits immunized only once (method IV) sometimes have ill-defined end points, and single tissue culture tubes may show viral cytopathogenic effect at a much higher serum concentration.
To make sure that this degeneration was not due to viraemia in the rabbits at the time of heart puncture, four of these sera were diluted in saline and inoculated into roller tubes. No cytopathogenic effect appeared in these control tubes.

Estimation of Serological Difference

In calculation of serological differences it is practical to use the following formula as employed for instance by *Wenner* (8).

$$\frac{\text{titre of serum 1 against virus 1}}{\text{titre of serum 1 against virus 2}} \times \frac{\text{titre of serum 2 against virus 2}}{\text{titre of serum 2 against virus 1}}$$

For identical strains this product is 1, for antigenic different strains it is >1. The calculations are facilitated when the above formula is transformed to logarithms on the base of 2.

$$D = \log_2 \frac{\text{titre of serum 1 against virus 1}}{\text{titre of serum 1 against virus 2}} + \log_2 \frac{\text{titre of serum 2 against virus 2}}{\text{titre of serum 2 against virus 1}} > 0$$

This procedure of calculating is reasonable as the

$$\log_2 \frac{\text{homologous titre}}{\text{heterologous titre}}$$

is distributed normally ($\chi = 1.85, f = 5, 0.80 < P < 0.90$).
Accordingly, in the text below the figures recorded are not the actual titres but their logarithms on the base of 2. D is a measure for antigenic difference.

TABLE 1

Serum Neutralization Titres Obtained in Groups of 6 Rabbits Immunized in 4 Different Ways with Mahoney or LSc 2ab Virus The Table Shows the Individual Titres Recorded as Logarithms on the Base of 2

The $\frac{\text{homologous titre}}{\text{heterologous titre}}$ is a measure for antibody specificity

Method of immunization	Rabbit number	Virus		Homologous titre / Heterologous titre
		Mahoney 125 TCID50	LSc 2ab 100 TCID50	
I (12 weeks) Mahoney	1	16	15	1
	2	15?*	16	−1
	3	13.5	13.5	0
	4	15.5	16	−0.5
	5	>16	14.5	>1.5
	6	>16	>16	–
I (12 weeks) LSc 2ab	7	13.5	16	2.5
	8	13.5	15	1.5
	9	14	>16	>2
	10	>16	>16	
	11	12.5	13.5	1
	12	12	15.5	3.5
II (8 weeks) Mahoney	13	12.5	<10	>2.5
	14	13.5	15	−1.5
	15	14	14	0
	16	11.5	12.5	−1
	17	13	13	0
	18	13.5	14	−0.5
II (8 weeks) LSc 2ab	19	11	14	3
	20	10.5	14.5	4
	21	14.5	16	1.5
	22	10	13	3
	23	13	13.5?	0.5
	24	13.5?	16	2.5
III (4 weeks) Mahoney	93	13	12.5	0.5
	94	10.5	11.5	−1
	95	11.5?	11	0.5
	96	11?	10	1
	107	12	12?	0
	108	9.5?	10.5	−1
III (4 weeks) LSc 2ab	85	10.5	11.5	1
	86	9	12	3
	87	11	13	2
	88	10.5	12	1.5
	89	10	14	4
	90	10	11.5	1.5
IV (1 week) Mahoney	97	11.5	10?	1.5
	98	9	8.5	0.5
	99	12.5	10.5	2
	100	9.5?	9?	0.5
	101	9.5?	10?	0.5
	102	13	8.5	4.5
IV (1 week) LSc 2ab	91	11.5?	13?	1.5
	92	8.5?	12.5	4
	103	8	13	5
	104	9?	11.5	2.5
	105	12?	13?	1
	106	10.5?	13?	2.5

As the neutralization titrations were repeated for the sera from rabbits immunized by method II and III $s_{\backslash}^2$ could be estimated

$$s_{\backslash}^2 = 0\ 37 \qquad s_{\backslash} = 0\ 61 \text{ (log}_e \text{ units)}$$

and consequently $\qquad s_{\backslash I}^2 + s_L^2 = 1\ 91$

CORRELATION BETWEEN SERUM TITERS AGAINST MAHONEY VIRUS AND LSc2ab VIRUS IN RABBITS IMMUNIZED WITH MAHONEY VIRUS OR LSc2ab VIRUS

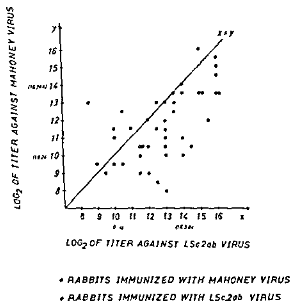


Fig 1

Plaque Neutrali-ation Tests

It was found of practical importance to examine whether sera produced within one week could be used for differentiation in plaque neutralization tests For these studies sera which had been found to have a high $\frac{\text{homologous titre}}{\text{heterologous titre}}$ in roller tube neutralization tests were chosen

The serum concentration required in the agar overlay to give a reasonably ... l to be between 10 and 80 antibody u ... variation supposedly being caused by ... of serum titres ($s_{\backslash}^2 = 0\ 37$, vide supra)

Table 3 illustrates an experiment of this kind The figures give the

TABLE 2

$$\log_2 \frac{\textit{Homologous Titre}}{\textit{Heterologous Titre}}$$

Means, m, Standard Deviations, s, and Mean Errors, e, of the Figures Listed in the last Column of Table 1

Method of immunization		I 12 weeks	II 8 weeks	III 4 weeks	IV 1 week
Virus Mahoney	m	0 20	−0 08	0 00	1.25
	s	1 04	1 39	0 83	1 89
	f	4	5	5	5
	e	0 58	0 70	0 42	0 95
LSc,2ab	m	2 10	2 42	2 17	2 75
	s	0 97	1 26	1 12	1 51
	f	4	5	5	5
	e	0 56	0 50	0 56	0 76
D=m(Mah)+m(LSc)		2 30	2 34	2 17	4 00
	s	1 42	1 86	1 40	2 41
	e	0 71	0 83	0 63	1 08
$t = \frac{D}{e}$		3 24	2 82	3 53	3 70
Number of rabbits necessary to demonstrate antigenic difference, D>0 (P<0 05)		2 × 2	3 × 2	2 × 2	2 × 2

The means, m, standard deviations, s, and mean errors, e, of

$$\log_2 \frac{\text{homologous titre}}{\text{heterologous titre}}$$

are listed in Table 2 It will be seen, that for all 4 methods of immunization the sum of the two means = D differs significantly from 0 Furthermore, the results show that this antigenic difference would be demonstrable using only 2 or 3 rabbits per virus strain instead of the 6 employed for each group in the present experiments

The standard deviations, s, of D only differ slightly for the methods of immunization (Barlett's test $\chi^2 = 1\ 65, f = 3, 0\ 30 < P < 0\ 40$ (*Hald* (3)), and therefore, a mean s_D is calculated

$s_D^2 = 3\ 39$ The mean of D is 2 70

s_D^2 is the sum of 4 times the variance on a neutralization titration in roller tubes s_N^2, and the variances in antibody specificity in rabbits immunized with Mahoney virus s_M^2 and with LSc 2ab virus s_L^2.

$$s_D^2 = s_M^2 + s_L^2 + 4s_N^2 = 3\ 39$$

To obtain more complete information one has however to examine sera prepared from the unknown virus strain with sera against known virus strains in cross neutralization tests It is therefore of practical value that the rabbit antisera which were collected one week after one solid virus dose appear to be as specific as the sera obtained after a longer immunization schedule and that the titres of such one week sera are satisfactory

It has been a general experience (*McBride* (4) *Nakano & Gelfand* (5)) that Mahoney antisera neutralize LSc 2ab virus and Mahoney virus to the same extent It was therefore surprising that two of the Mahoney antisera (Nos 13 and 102) from the present experiments were found to be very Mahoney specific This specificity was not due to Mahoney antibodies in the two rabbits before immunization since a 1 2 dilution of serum collected before the immunization was not able to neutralize 25 $TCID_{50}$ Mahoney virus in roller tubes

Specific sera of this kind are of course very precious for antigenic analysis as it is evident from Table 3 In the plaque neutralization experiment recorded here a good differentiation was obtained using only the number of plaques as did *Nakano & Gelfand* (5) Also the size of the plaques can be used and may give a sharper differentiation even if number and size seem to be rather closely correlated (*Wecker* (7))

In a new series of experiments the 1 week immunization technique was used (unpublished data) In these experiments the antigenic difference between the Mahoney and LSc 2ab viruses was demonstrable roller tube neutralization tests as clearly as in the experiments recorded here ($\bar{D}$ = 2 33 $s^2_{\bar{D}}$ 1 475 t = 4 66) but none of the six new Mahoney antisera were as Mahoney specific as sera 13 and 102

Antigenic analysis of wild poliovirus strains using the 1 week immunization method for immunization of rabbits is in progress

SUMMARY

Groups of 6 rabbits were immunized with the Mahoney and LSc 2ab polioviruses using 4 different dosage schemes The antibody titres were found to increase with the length of the immunization period However the four methods of immunization resulted in sera equally suitable for distinguishing one virus strain form the other in cross neutralization tests in roller tubes For epidemiological use it is therefore suggested to employ sera produced in one week after the injection of 10^8 10^9 $TCID_{50}$ In plaque neutralization tests such 1 week sera gave a satisfactory degree of differentiation

Mahoney antisera are usually observed to neutralize Mahoney virus and LSc 2ab virus to the same extent but among the 24 Mahoney sera produced in the present experiments two contained Mahoney specific antibodies Pre immunization sera from these two rabbits contained no antibodies against Mahoney virus

TABLE 3

Plaque Neutralization Tests (37°) Using 1 Week Sera Against Mahoney Virus LSc,2ab Virus and a Strain 10077IV from a Vaccinated Person

	Virus					
	Mahoney		LSc,2ab		10077IV	
Experiment read day	2	3	2	3	2	3
Virus control	13×10	18×10	12×10	18×10	39×10	4×10
Serum						
Mahoney Rabbit 102						
40 AU	0	9	22	58	74	137
20 AU	30	44	49	86	80	114
LSc,2ab Rabbit 103						
40 AU	40	65	0	2	10	19
20 AU	55	117	12	23	32	86

number of plaques on the last day when Mahoney plaques had not yet appeared in the bottle with Mahoney serum, and also the readings from the following day Incorporated in the experiment was a type 1 poliovirus strain, 10077IV, isolated from a healthy child 3 weeks after oral vaccination with LSc,2ab It may be seen that bottles with 40 AU per 0 2 ml agar overlay demonstrated the antigenic difference If 20 AU per 0 2 ml had been the only concentration used, the difference might not have been demonstrable The faecal strain appears to be antigenically different from the Mahoney strain but similar to the LSc,2ab, as it is suppressed to about the same degree as the LSc,2ab strain both by Mahoney antiserum 102 and by LSc,2ab antiserum 103

In plaque tests not included in the present report the six 1-week Mahoney sera inhibited Mahoney virus at least to the same extent as LSc,2ab virus Of the six 1-week LSc,2ab sera, four (Nos 92 103 104–105) inhibited LSc,2ab virus at a *dilution not inhibiting* Mahoney virus, but two (Nos 91–106) gave no differentiation

DISCUSSION

The antigenic specificity of poliovirus subtypes is a useful and rather stable marker The differentiation may be carried out using known specific antisera, the degree of neutralization of known virus strains being compared to the neutralization of the virus isolated from a patient

To obtain more complete information one has however to examine sera prepared from the unknown virus strain with sera against known virus strains in cross neutralization tests. It is therefore of practical value that the rabbit antisera which were collected one week after one solid virus dose appear to be as specific as the sera obtained after a longer immunization schedule and that the titres of such one week-sera are satisfactory.

It has been a general experience (*McBride* (4), *Nakano & Gelfand* (5)) that Mahoney antisera neutralize LSc 2ab virus and Mahoney virus to the same extent. It was therefore surprising that two of the Mahoney antisera (Nos. 13 and 102) from the present experiments were found to be very Mahoney specific. This specificity was not due to Mahoney antibodies in the two rabbits before immunization since a 1:2 dilution of serum collected before the immunization was not able to neutralize 25 $TCID_{50}$ Mahoney virus in roller tubes.

Specific sera of this kind are of course very precious for antigenic analysis as it is evident from Table 3. In the plaque neutralization experiment recorded here a good differentiation was obtained using only the number of plaques as did *Nakano & Gelfand* (5). Also the size of the plaques can be used and may give a sharper differentiation even if number and size seem to be rather closely correlated (*Wecker* (7)).

In a new series of experiments the 1 week immunization technique was used (unpublished data). In these experiments the antigenic difference between the Mahoney and LSc 2ab viruses was demonstrable roller tube neutralization tests as clearly as in the experiments recorded here ($D = 2.33$; $s^2_D = 1.475$; $t = 4.66$) but none of the six new Mahoney antisera were as Mahoney specific as sera 13 and 102.

Antigenic analysis of wild poliovirus strains using the 1 week immunization method for immunization of rabbits is in progress.

SUMMARY

Groups of 6 rabbits were immunized with the Mahoney and LSc 2ab polioviruses using 4 different dosage schemes. The antibody titres were found to increase with the length of the immunization period. However, the four methods of immunization resulted in sera equally suitable for distinguishing one virus strain form the other in cross neutralization tests in roller tubes. For epidemiological use it is therefore suggested to employ sera produced in one week after the injection of 10^8–10^9 $TCID_{50}$. In plaque neutralization tests such 1 week sera gave a satisfactory degree of differentiation.

Mahoney antisera are usually observed to neutralize Mahoney virus and LSc 2ab virus to the same extent, but among the 24 Mahoney sera produced in the present experiments two contained Mahoney specific antibodies. Pre-immunization sera from these two rabbits contained no antibodies against Mahoney virus.

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TRANSACTIONS
OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting December 1–2, 1961

Received 09 ii 63

1 *M Nasiell* THE GENERAL APPEARANCE OF THE BRONCHIAL MUCOSA IN BRONCHIAL CANCER

The bronchial mucosa has been investigated in 50 cases of bronchial carcinoma and 56 control cases Basal cell hyperplasia was often found in both series A similar alteration (pretransitional epithelium) and also slit formation and expulsion" were however more common in the cancer material A slit is formed underneath the surface cells resulting in a low epithelium without cilia This probably provides the histopathological explanation for Papanicolaous Ciliocytophthoria Keratinized squamous epithelium was found in carcinoma cases only and always within the tumour Papilloma like stromaproliferations (micropapilloma") had occurred in 60 and 25 per cent respectively of the cases while metaplastic epithelium (formed via basal cell hyperplasia or low epithelium) was found in 80 and 34 per cent respectively Advanced epithelial atypia and extensive metaplasia was observed only in the cancer series and the former only in connection with transitional meta plasia This suggested the existence of a generalized epithelial disturbance in the cancer cases

2 *Th Berge* and *O Grontoft* CYTOLOGICAL DIAGNOSIS OF PLEURAL MESOTHELIOMA

3 *O Grontoft* HISTO PATHOLOGICAL DIAGNOSIS OF NEEDLE BIOPSIES FROM THE PROSTATE GLAND (prel communication)

4 *A Bäckgren L E Johansson A Norden S Rubarth* and *G Winqvist* SPONTANEOUS LEUCOSIS IN DOGS

In S

... years of age The mean age was 6 7 years The *mean age for* the whole autopsy material during this period was 4 32 years Sex distribution of the whole material was 51 5 per cent males and 48 5 per cent females In the leucosis material 58 8 per cent *were* males and 41 2 per cent females The disease seems to be predominant in males this is however not significant As carcinoma in dogs leucosis appears most frequently in boxers and in Airedale terriers In connection with the advanced mean age this perhaps may be an expression for the blastomatous character of leucosis

5 *B Lagerlöf* TRANSPLANTATION OF CHROMOSOME LABELLED LEUKAEMIA CELLS

A transplantation form of virus induced erythroleukaemia in the fowl can be obtained by inoculating the chick with intact leukaemia cells The host s own bone marrow cells which are the target for the virus, may, however be infected by the virus released from the leukaemia cells and thereby undergo leukaemic transformation In its final stage the disease thus developed would then be a combination of *transplantation* and virus induced leukaemia

The occurrence of transplanted and autochthonous leukaemia cells in the body has been studied by chromosome analysis Hens the cells of which have 11 macro chromosomes and an unpaired fifth chromosome were inoculated with leukaemia cells of cocks which have 12 macro chromosomes and paired fifth chromosomes Three and four days after such inoculation the animals showed varying degrees of transplanted leukaemic proliferation in the bone marrow, and in the spleens the infiltration was in most cases due entirely to the proliferation of the transplanted *cells On the fifth day a few of the host s own cells were also recognized in the* spleen and the bone marrow contained a mixture of autochthonous and transplanted leukaemic proliferation It was concluded that during the first days the animals can develop a fairly pure transplantation leukaemia on which a virus induced component is subsequently super imposed

6 *G Hultquist* and *J Thorell* TRIALS WITH PANCREAS TRANSPLANTATION IN RATS AND GUINEA PIGS

Pancreas tissue from rats or guinea pigs with pancreatic duct ligation and containing islet tissue but no other remains of exocrine parenchyma except ducts was implanted in the anterior chamber of the eye The transplantations were carried out both in the animals with duct ligation and in other members of the same litter After one month islet tissue showing signs of growth was found in many of the rats about *90 per cent of the case of autologous transplantation and about* 35 per cent of the others Much poorer results were shown by the guinea pigs in which the survival time was 9 10 days at the maximum

The grafts always contained β cells showing a pseudo isocyanine reaction *but* α cells were present only in some cases In a few α cells a positive reaction was obtained with Voigt s sulfide silver method

Karyometry showed nuclear enlargement in both β and α cells with maximum enlargement in *3 4* day grafts The change gradually decreased and after 3 4 weeks the values corresponded to those of the normal pancreas

7 *G Nathorst Windahl* DIABETES IN PANCREATECTOMIZED RABBITS

Subtotal pancreatectomy in rabbits produces a relatively mild type of diabetes with glycosuria polyuria hyperglycaemia and an initial loss of weight There is often an overproduction of ketone bodies but severe acidosis or coma does not develop The animals are able to live for long periods without insulin and yet often maintain an almost constant rather low body weight in spite of pronounced *glycosuria*

Total or approximately total pancreastetomy however produces a severe diabetes with massive ketonaemia and ketonuria Metabolic acidosis and coma develop in a few days unless *insulin is given*

These results are at variance with the opinion of Greeley as cited by Houssay

(Comparative Endocrinology, Wiley, New York 1959) who stated that total pancreatectomy produces a mild type of diabetes in rabbits

The pancreatic rests in the subtotally depancreatized diabetic rabbits have been studied Pictures suggestive but not convincing of so called acino insular transformation have been obtained Glycogen infiltration in hydropic β cells has been shown

8 *S Folkmer* and *F Knutson* IS COBALT CONCENTRATED IN PANCREATIC ISLET TISSUE?

9 *I Zamboni* ELECTRON MICROSCOPICAL INVESTIGATION ON THE EARLY STAGES OF POLYOMA VIRUS INFECTION IN MICE

A pellet obtained by ultracentrifugation of medium from polyoma infected tissue culture and very rich in virus particles mostly attached to fragments of tissue culture cells was resuspended in a small quantity of fluid and subcutaneously injected in adult mice

15 minutes 45 minutes and 24 hours later subcutaneous tissue was excised from the injection area and studied with the Electron Microscope 15 minutes after the injection virus particles were observed in the cytoplasm of the tissue cells They had been transported through the cell membrane as single units by pinocytotic activity or in clusters attached to the injected cell fragments by phagocytosis

After 45 minutes few particles were detectable intranuclearly whereas 24 hours later the nuclei of many cells were litterally loaded with virus The degeneration of the infected cells leads to a fragmentation of the elements their debris rich in virus particles are phagocyted by healthy cells with consequential spreading of the infection

10 *A Bergstrand* *C G Bergstrand* *N Engstrom* and *K M Herrlin* RENAL CHANGES DURING TRIDIONE TREATMENT

A nephrotic syndrome is a rare complication to treatment of epilepsy in children with Tridione ® Experimental investigations also indicate that Tridione in large doses may have a nephrotoxic effect In order to find out if slight and clinically non observed damage to the kidneys is a frequent complication we have investigated seven cases aged 15 17 years which have been treated with Tridione for periods between three months and eights years Continuous renal examination showed only slight and transient haematuria or proteinuria in three of the cases

Light microscopy of renal needle biopsy material showed no glomerular or tubular lesions

Electron microscopy of the glomeruli showed wide variations in the appearance of the capillary epithelial cells These are regarded as physiological variations and not as pathological phenomena

The investigation has not shwon any signs of renal damage which could be assigned to the treatment

11 *B Thorell* LOCALIZATION AND DETERMINATION OF RESPIRATORY ENZYMES IN THE SINGLE LIVING CELL

With regard to the fundamental energy linked cell reactions e g the cell respiration our knowledge about the functions of the various cell structures has

so far mainly been based on bulk analyses of isaloted tissue fractions The present paper describes some recent developments *of the microspectrographic technique* which permit quantitative *in vivo* assay of respiratory enzymes in localized areas of 1 5 μ in diameter in the single intact cell The detectability *is equivalent to 6000* molecules of cytochrome *b* Soret spectra of both oxidised and reduced cytochromes can be demonstrated depending on whether the cell is in an aerobic or an anaerobic state Measurements on the grasshopper spermatocyte and on isolated liver and kidney cells have given information about the metabolic state of the respiratory system even of the single structures within the living cell

12 *K F Aström* EXPERIMENTS ON ALLERGIC ENCEPHALOMYELITIS

13 *S S-ogi* ASEPTIC MENINGOENCEPHALITIS AND MYOCARDITIS (PROBABLY COCKSACKIE) IN A NEW BORN CHILD

14 *S Lundberg* and *O Grontoft* A CASE OF BILATERAL SYMMETRICAL CALCIFICATIONS OF THE BRAIN

15 *L Bjersing* and *A E Borglin* EOSINOPHILIA IN UTERUS

In recent years the authors have occasionally seen massive collections of eosinophilic leucocytes in the human uterine myometrium Fifteen cases were studied in detail Two groups were used for comparison In one of the groups amputation of the uterus or cervix had been preceded by curettage or biopsy but not in the other Eosinophilic granulocytes were observed—also in the controls—usually around vessels or between muscle bundles in the myometrium and showed no correlation with the amout or site of other inflammatory cells No explanation can be offered for this abnormal eosinophilia which has as far as we know not been *described* before The eosinophilia was slight and it was rare when the interval between the *interventions was more than 3 weeks Animal experiments in progress* have shown absence of eosinophilic leucocytes in the uterus of spayed rats and eosinophilia in spayed animals treated with oestrogen the degree of eosinophilia varying closely with the dose given

16 *G Fichera* and *I Hagerstrand* LYMPHATIC CHANGES IN CHRONIC PULMONARY CONGESTION

A post mortem series of chronic pulmonary congestion (100 cases of mitral *stenosis 75 cases of pulmonary* congestion because of hypertension or myocardial lesions) was studied Compared with the controls (healthy lungs from 50 cases of different ages) fibrous mural thickening of the lymphatics was found at different levels (perivascular peribronchial interlobular and intrapleural) in 70 per cent of the cases of pulmonary congestion This lymphatic fibrosis appeared to develop together with other pathological signs of chronic congestion and was probably *not* a primary lesion The fibrosis was probably due to increased drainage of fluid

17 *I Hagerstrand* and *F Linell* SARCOIDOSIS AND POLLEN

Experiments were performed to check the hypothesis put forward in recent years that sarcoidosis might be due to fir tree pollen All of the pollen studied (different sorts of fir tree pine beech oak maple alder lycopodium *timothy grass*) proved

acid fast Pollen was suspended in physiological saline and injected subcutaneously and intraperitoneally into rabbits (33 animals) The animals were killed 1 8 months later Foreign body granulomas developed around the injections but no epithelioid cell granulomatosis was observed The experiments provided no support to the hypothesis of a causal relationship between pollen and sarcoidosis

18 *L E Larsson* ENDOCARDIAL MYXOMA WITH MALIGNANT DEGENERATION

A woman without known rheumatic fever had for 3 years had progressive cardiac failure clinically interpreted as combined mitral and aortic disease During the last few months widespread skeletal lesions of malignant character were observed Necropsy revealed a myxomatous tumour at the fossa ovalis in the left atrium The endocardium in the atrium was thickened the mitral orifice was stenosed the mitral valves and chordae tendinae were rigid and thick and the aortic valves showed diffuse fibrous thickening The left lung showed a hilar tumourous nodule with peripheral extensions along the vessels Numerous metastases were seen in the heart wall in the skeleton and in the left ovary The atrial tumour exhibited the histological characteristics of an endocardial myxoma with sarcomatous degeneration The rest of the atrial wall the mitral and aortic valves chordae tendinae and the pulmonary veins showed marked fibrous thickening with a surface layer of anaplastic tumour cells The relations between the tumour and the valvular and pulmonary lesions are discussed

19 *L Olding* PERINATAL BACTERIAL INFECTIONS IN AN AUTOPSY MATERIAL

20 *C Lundmark* GRANULAR ATROPHY OF THE BRAIN—OBLITERATING ARTERIAL DISEASE

21 *C Lundmark* CARCINOID TUMOUR WITH METASTASES IN THE HEART AND AORTIC STENOSIS

22 *B Larsson* A CASE OF POSTANGIOGRAPHIC Th DEPOSITION AND PRIMARY SQUAMOUS CELL CARCINOMA OF THE LIVER

A bilateral cerebral angiography had been performed with a total of 17 ml of Thorotrast Twenty seven years later the 53 year old male succombed to a large necrotizing liver tumour squamous cell in type Au^{198} total body scintillography had shown no uptake in the region of the spleen before a surgical splenectomy Metastases were found in regional lymph nodes the peritoneum the adrenal regions and in one thoracic vertebra There were no thorium dioxide deposits at the sites of injection The liver weighed 1800 g and revealed a moderate and mainly post necrotic scarring Thorotrast *deposition was demonstrated in* liver and spleen with routine methods and autoradiography The bone marrow was in a reactive state A moderate anaemia was present There were no oesophageal varices

TRANSACTIONS
OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting April 7, 1962

1 *L-B Schnürer* and *S Stattin* THE VASCULAR SUPPLY OF TENTORIAL MENINGIOMAS

In some papers dealing with tentorial meningiomas vessels have been described seemingly arising from the carotid siphon This prompted an investigation on the topography and distribution of these vessels, performed on autopsy specimens with the aid of a dissection microscope The vessels were successfully injected and dissected in about 15 of the specimens The number and course of the major branches were found to be rather constant Thus there are two main branches one arising from the infero-lateral aspect of the so called horizontal portion of the siphon the other from the upper posterior aspect of the convexity of the proximal portion of the siphon In most cases both main stems give off tentorial branches in some only one of them does The tentorial branch of the lateral main stem in most cases runs directly upward enters the tentorium and continues backward near the free margin sometimes up to the summit The tentorial branch of the dorsal main stem is usually smaller and runs backward in the tentorium near its attachment to the petrous bone These tentorial vessels are most often but not always too small to be visualized at angiography, but they may hypertrophy when contributing to the vascular supply of meningiomas and vascular malformations and thus be of angiographic and neurosurgical importance

2 *C Ahrén* EFFECT OF DIENCEPHALIC LESIONS ON TESTIS IN ADULT RABBITS

Diencephalic lesions were produced in 248 adult male rabbits with the aid of Ranstrom s X ray stereotaxic instrument in medial parts of the diencephalon from the anterior medial preoptic area to the rostral border of the mesencephalon The animals were allowed to survive for 6 weeks, half of these were subjected to repeated subcutaneous injections of formalin during the final 3 weeks On the basis of the size localization and symmetry of the lesions determined histologically from serial sections of the diencephalon 140 animals were segregated into 10 groups (Each about equally divided as to formalin treatment) The median eminence and pituitary gland were histologically intact in all animals

Results Some animals showed gross testicular atrophy not correlated to any particular lesion Severe atrophy was only observed with formalin treatment probably a non specific stress reaction There were no significant differences in the mean testicular weight in the 10 groups as compared with the control animals Histological signs of altered spermatogenesis in some animals suggest disturbances in secretion of FSH Such changes were most frequently observed in animals with

posterior diencephalic lesions However, these results are preliminary and must be confirmed by further studies

The results support the view that there is no localized "sexual center" in the adult male rabbit However posterior diencephalic lesions seem to induce alterations in spermatogenesis

2 *P Sourander* MORBUS KRABBE

4 *I Hagerstrand* THALIDOMID INDUCED MALFORMATIONS

5 *H Sunzel* and *L Zettergren* LESIONS IN THE LIVER AFTER SURGICAL OPERATIONS IN THE UPPER PART OF THE ABDOMEN

6 *L Faerbäck* HISTO CHEMICAL REACTIONS IN CARCINOIDS

7 *F Knutson* AUTORADIOGRAPHY OF PITUITARY CELLS IN ADRENAL-ECTOMIZED WHITE RATS AFTER THE INJECTION OF ADENINE-^{14}C

Five male white rats weighing 150–200 gramts were adrenalectomized Observation times were 1 2 4 8 and 12 days Two hours before the end of each observation time the animals were injected with 0 05 mC adenine ^{14}C.

The pituitaries were fixed in neutral formalin embedded in paraffin and sectioned serially Autoradiography was performed with strip film technique and Kodak AR 10 emulsion The unstained autoradiographic specimen was mounted in a glycerol medium and examined under a photo microscope with a micron graduated mechanical stage Labelled cells were photographed in transmitted light and in phase contrast The the film was removed and the section stained with Tri PAS technique With the aid of the graduated stage and the previously taken photographs the same labelled cells were localized in the stained section

About 70 labelled cells within random fields in all pituitaries had been so localized One day after adrenalectomy 70 per cent of labelled cells were small chromophobes With increasing time after adrenalectomy their percentage gradually diminished whereas the number of labelled large vacuolized chromophobes and amphophiles increased reaching almost 50 per cent 8 and 12 days after adrenalectomy Very few well granulated basophiles were labelled The results point at the chromophobe cell as the site of new production of ACTH and it was suggested that these chromophobes gradually turned into vacuolized forms and so called amphophiles

8 *P Juntin* THE CARCINOGENIC ACTION OF COMPLEX IRON PREPARATIONS

9 *H Röckert* and *L Zettergren* LESIONS CAUSED BY BARIUM CONTRAST MEDIA

10 *I Angervall* and *S E Fagerberg* SKIN BIOPSY IN DIABETES

Since the beginning of 1961 punch biopsies from the skin on the dorsal part of the basal phalanx of the 4th toe of diabetics have been examined All specimens have been fixed in 10 per cent neutral formalin solution about half of them being

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fixed also in 4 per cent basic lead acetate solution The skin specimens were cut into 4 μ serial sections consecutive sections being stained with haematoxylin eosine van Gieson haematoxylin Hansen and MacManus periodic acid Schiff So far we have examined biopsy specimens from 50 male and female diabetics of varying ages and from 35 non diabetics

All but one of the diabetics exhibited dermal microangiopathy in the form of wall thickening and reduction in the lumen due to deposition of a strongly PAS positive substance which in van Gieson stained sections was a homogeneous mildly picrinophilic materal Some vessels displayed hypertrophic hyperchromatic nuclei between PAS positive fibrils of the basement membrane The diabetic microangiopathy is usually most pronounced in the capillaries surrounding the sweat glands and in the papillae The larger the vessels the more difficult it is to find characteristic lesions

All but one of the controls showed in general a suggested or slight PAS positive reaction

The facts that diabetic microangiopathy has now been encountered in the skin constitutes additional evidence that it occurs throughout the organism

TRANSACTIONS
OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting December 1, 1962

1 *L Santesson* ON THE CLASSIFICATION OF OVARIAN TUMOURS

At an international conference in Stockholm the following proposal was made
The Conference recommends that the common epithelial tumours of the ovary be classified in the following manner

1 *Serous cystomas*
  a) Serous papillary cystadenoma benign (Figs 69 and 71)
  b) Proliferating serous papillary cystadenoma without stromal invasion (possibly malignant) (Figs 72 and 75)
  c) Serous cystadenocarcinoma all grades (Figs 78 and 81)
2 *Mucinous cystomas*
  a) Mucinous cystadenoma benign (Figs 87 and 90)
  b) Proliferating mucinous cystadenoma without stromal invasion(possibly malignant) (Figs 91 and 92)
  c) Mucinous cystadenocarcinoma all grades (Figs 93 and 95)
3 *Endometrioid tumours*
  a) [1]
  b) Proliferating endometrioid adenoma and cystadenoma (possibly malignant) (Fig 102)
  c) Endometrioid adenocarcinoma all grades (Figs 77 and 105)
4 *Undifferentiated carcinoma* (cell type unknown)

(The numbers in parenthesis refer to figures published in Tumors of the Ovary and Fallopian Tube by *Hertig A T & Gore H* (Atlas of Tumor Pathology Section IX Fascicle 33 Tumors of the Female Sex Organs Part 3 Armed Forces Institute of Pathology Washington 1961))

Usually in any given tumour only one type of epithelial cell is dominating. Occasionally when there is a mixture of different cell types the predominant type should determine the diagnosis

2 *S Falkmer F Knutson* and *G E Voigt* FURTHER STUDIES ON THE COBALT CONCENTRATING *ABILITY OF PANCREATIC ISLET TISSUE*

The recent discovery by quantitative scintillation determinations using ^{58}Co that isolated islet tissue has approximately the same high ability as the liver to con

[1] The existence of a blank space after 3 a) is due to the fact that there is as yet no agreement as to whether some cases of ovarian endometriosis may properly be classed as true tumours or all must be regarded as simply displaced or ectopic endometrium

centrate cobalt (Acta Endocr 42 1963) prompted us to try further localization of the cobalt in the pancreatic islets As conventional autoradiography had shown that the accumulated cobalt was easily dissolved from the tissue during fixation and embedding procedures we had to modify the histochemical and autoradiographical methods

The isolated islet tissue of the marine teleost *Cottus scorpius* was cut in a microtome cryostat Autoradiography was performed without the sections coming in contact with water Other islets were gassed with hydrogen sulphide sectioned in the microtome cryostat and subjected to a modified sulphide silver procedure to detect precipitated cobaltous sulphide after extracting other heavy metal sulphides The injected cobalt was localized to the islet parenchyma and not to the stroma By comparing the autoradiograms and the sulphide-silver sections with adjacent sections stained with aldehyde fuchsin (for the visualisation of the B- and A cells) it was possible to show that the injected cobalt was confined only to the cells in the dark central region of the sculpin islets containing the B cells and the argyrophil A cells

3 *B Stenkvist* GROWTH CURVES HISTOLOGICAL APPEARANCE AND VIRAL TITRES OF ROUS SARCOMAS A COMPARISON BETWEEN PROGRESSIVE AND REGRESSIVE GROWING TUMOURS

4 *J Thorell* THE DIAPLACENTAL TRANSFER OF INSULIN ANTIBODIES

The insulin antibody titre was assayed by measuring the capacity of serum to bind insulin-I^{131} After incubating the sera with insulin-I^{131} for 5 days at 4° C the antibody-bound insulin was separated from the unbound insulin by paper electrophoresis according to *Berson & Yalow* The antibody titres were assayed in blood obtained from the umbilical cord and from the mother on the same occasion

In pregnant guinea pigs with relatively low insulin antibody titres the insulin binding capacity of the foetal blood was usually double that of the maternal blood at term

The titres of three newborn children of insulin trated diabetic mothers were about equal to their mothers titres

This placental transfer of antibodies may be of some importance for the foetal insulin metabolism Further studies on this subject are in progress

5 *P Sundelin* and *B Lagerlof* DISEASES INDUCED BY MYELOID LEUKAEMIA VIRUS

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chicks with a late onset of the disease dying at an age of more than one month had a significant increase of the gamma globulin fraction This probably contains viral antibodies and may thus be responsible for the fact that the virus concentration of the plasma from leukaemic chicks decreases with increasing age of the birds Young chicks with marked viraemia had normal amounts of gamma globulin Chicks even young ones with the subleukaemic anaemia had high gamma globulin fractions which taken together with the lymphoid hyperplasia and the signs of haemolysis seems to indicate an immunologic cause of the disease

6 *T Salleen* EXPERIMENTAL INVESTIGATION OF CANCER SPREAD TO THE LIVER IN RATS WITH LIVER CIRRHOSIS

Moderate liver cirrhosis induced by administration of carbon tetrachloride to rats increased the percentage of secondary intrahepatic growths following intraportal injection of tumour cells from Rous rat sarcoma The number of tumour nodules in the liver was higher when the interval between the last injection of carbon tetrachloride and the injection of tumour cells was 4 or 6 weeks than when it was 2 weeks A positive correlation was found between the extent of fibrosis in the liver and the number of tumour nodules

After injection of tumour cells into a tail vein tumour nodules were seen in most cirrhotic but in no normal livers

Liver cirrhosis had no effect on the growth of the tumour after direct intrahepatic injection of the tumour cells suggesting that the vascular changes in the liver are responsible for the increased number of intrahepatic secondary growths

A possible increase in the number of tumour cells trapped in the cirrhotic livers can however hardly explain the higher percentage of takes in the liver Another possibility is that the narrowing and obliteration of the smaller vessels prevented the tumour cells from coming into contact with Kupffers cells

7 *J Zajicek* NEEDLE BIOPSIES AS A METHOD TO OBTAIN HUMAN TUMOUR MATERIAL FOR BIOCHEMICAL AND BIOLOGICAL INVESTIGATIONS

8 *P S Persson* CYTOLOGIC DIAGNOSIS OF SUBACUTE AND CHRONIC THYROIDITIS

Thin needle punctures were carried out in 175 patients with goitres The smears were stained according to the May Grunewald Giemsa method Of 9 patients with the clinical picture of subacute thyroiditis 6 had the cytologic findings of subacute thyroiditis and 2 a suspected one No definite diagnosis could be made in 1 patient In 42 patients the cytologic diagnosis was chronic thyroiditis In 24 patients the diagnosis was verified by histopathological examination the rest had a typical clinical picture There were no cytologic differences between various kinds of chronic thyroiditis

The cytologic findings of subacute thyroiditis were [illegible]
a [illegible]
t [illegible]
t [illegible] collections of polymorphonuclear leucocytes histiocytes and mononuclear inflammatory cells

The cytology of chronic thyroiditis is characterized by (1) proliferation and to a lower degree (2) degeneration of the follicular epithelial cells (3) collections

centrate cobalt (Acta Endocr 42 1963) prompted us to try further localization of the cobalt in the pancreatic islets As conventional autoradiography had shown that the accumulated cobalt was easily dissolved from the tissue during fixation and embedding procedures we had to modify the histochemical and autoradiographical methods

The isolated islet tissue of the marine teleost *Cottus scorpius* was cut in a microtome cryostat Autoradiography was performed without the sections coming in contact with water Other islets were gassed with hydrogen sulphide sectioned in the microtome cryostat and subjected to a modified sulphide silver procedure to detect precipitated cobaltous sulphide after extracting other heavy metal sulphides The injected cobalt was localized to the islet parenchyma and not to the stroma By comparing the autoradiograms and the sulphide-silver sections with adjacent sections stained with aldehyde fuchsin (for the visualisation of the B- and A-cells) it was possible to show that the injected cobalt was confined only to the cells in the dark central region of the sculpin islets containing the B cells and the argyrophil A cells

3 *B Stenkvist* GROWTH CURVES HISTOLOGICAL APPEARANCE AND VIRAL TITRES OF ROUS SARCOMAS A COMPARISON BETWEEN PROGRESSIVE AND REGRESSIVE GROWING TUMOURS

4 *J Thorell* THE DIAPLACENTAL TRANSFER OF INSULIN ANTIBODIES

The insulin antibody titre was assayed by measuring the capacity of serum to bind insulin I[131] After incubating the sera with insulin-I[131] for 5 days at 4° C the antibody bound insulin was separated from the unbound insulin by paper electrophoresis according to *Berson & Yalow* The antibody titres were assayed in blood obtained from the umbilical cord and from the mother on the same occasion

In pregnant guinea pigs with relatively low insulin-antibody titres the insulin binding capacity of the foetal blood was usually double that of the maternal blood at term

The titres of three newborn children of insulin trated diabetic mothers were about equal to their mothers titres

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The cytol gic findings of subacute thyroiditis vary with the phase of the disease and present the f llowing features (1) marked degeneration of the follicular epithelial cells (2) proliferation of the follicular epithelial cells in the late phase of the d sease (3) foreign body giant cells (4) collections of polymorphonuclear leucocytes histiocytes and mononuclear inflammatory cells

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11 *A Cederberg* and *G Östberg* OXYGEN INTOXICATION

A girl of eighteen after some days of sore throat suddenly developed pain in the right side of the chest and heavy respiratory distress On the third day she was taken to hospital where blood was found in the right pleura She was given transfusions and treated with drainage of pleura later on tracheotomy and artificial respiration with increasing partiel pressure of oxygen up to 52 per cent in the last hours She died on the tenth day Clinical diagnosis Spontaneous haemo pneumothorax

At autopsy the lungs which weighed 830 and 870 grams were strikingly heavy and firm No rupture or bleeding was found on the lung surface Microscopically a dense fibrinous exudate filling the alveoli of right lower lobe was seen Other parts showed widespread formation of hyaline membranes on alveolar septa

Pulmonary hyaline membranes of adults are rare They seem to occur without any certain relation to other diseases The rôle of oxygen treatment has been discussed Possibly hyaline membrane formation can be induced by thoracic surgery Our case might have a similar mechanism with collapse of the right lung and compression of the left at spontaneous haemo-pneumothorax Delay of treatment may have played a role

12 *J Söderström* SPLENIC RUPTURE IN INFECTIOUS MONONUCLEOSIS

About 50 cases of splenic rupture in infectious mononucleosis are reported The histological picture is described on the basis of a personal collection of 6 cases Three of the 6 patients had died and 3 had been operated upon with success The spleen showed intense proliferation of large mononuclear cells above all in the red marrow but also in the capsule and the trabecular network that had thereby become loose which probably facilitated the rupture The cellular proliferation in the vessels was noteworthy Large round cells were seen in the adventitia of the arteries and subintimally in the veins Other organs showed similar changes The picture was the same in all the cases studied and coincided with descriptions of earlier reported cases

The most important disease to be considered in the differential diagnosis is leucosis Careful pathological examination of the characteristic—though not pathognomonic—histological picture together with clinical and laboratory data enable a firm diagnosis of mononucleosis infectiosa

13 *A Rausing* GLIOMA INDUCED BY FOREIGN BODY

A 55 year old woman with bilateral symptoms of parkinsonism since 1951 1956 pallidotomy right side leaving two spirals of tantal in the brain 1959 left sided thalamotomy no foreign bodies left behind No relapse on left side speedy recurrence on right side and progressing symptoms of increased intracranial pressure until death in 1962

Autopsy small-orange sized cyst in right sided field of operation Two metal spirals were found in its wall Walnut sized tumour in the right occipital lobe Brownish discoloration in the left sided field of operation *Histology* tissue from right sided cyst wall and tumour and left sided lesion showing malignant glioma No continuity proved between the different sites [illegible]

[illegible] ng spindle-cell sarcomas by subcutaneous

of lymphocytes lymphoblasts plasma cells reticulo endothelial stem cells and phagocytes Epitheloid cells may sometimes be seen

Difficulties in the differential diagnosis exist between thyroiditis and special types of thyrotoxicosis and malignant thyroid diseases

9 *L G Wiman* SULPHYDRYL CHEMISTRY AND FLUORESCENCE MICROSCOPY IN EXFOLIATIVE PULMONARY CYTOLOGY

Two new cytochemical staining procedures for exfoliative cytology are introduced With one method first published in 1959 by the author a high concentration of proteinbound sulphydryl groups is demonstrated in exfoliated tumour cells In ordinary light microscopy these cells stain intensive blue and can be easily distinguished from surrounding normal cells which appear as pale shadows With a second method (1962) the ability of tetracycline to fluoresce in ultraviolet light is used to display tumour cells In a patient treated with tetracycline the substance is concentrated in tumours especially According to their conspicuous yellow green fluorescence exfoliated malignant cells can be easily identified in smears of sputum bronchial secretions aspirates from pulmonary tumours and pleural fluids If unfixed smears from patients not treated with tetracycline are immersed in a tetracycline solution a strong yellow fluorescence is induced in the tumour cells Upon the same material Bertalanffy s method was tried using fluorescence microscopy after staining with acridine orange Hereby certain malignant cells got a bright red fluorescence on account of a high content of RNA in cytoplasm and nucleolus which makes them easy to find during screening work However malignant cells exfoliated from a highly differentiated squamous cell carcinoma often have a low content of RNA For this reason they get a faint olive green or orange red fluorescence and may therefore be overlooked in the microscope This and some practical disadvantages gives the method a limited value in diagnostic cytology The results of all methods above has been correlated to and compared with the Papanicolaou staining procedure

10 *K Gydell I Juhlin* and *J G Norden* NOCARDIOSIS

A 41 year old female acquired an auto immunologic haemolytic anaemia and was treated with increasing doses of steroids (average dose during 1 year 29 mg of Deltacortril® per day) After 6 months she got fever and chest roentgen showed infiltrations which later cavitated No acid fast bacilli were found guinea pig inoculation was negative but nocardia was cultivated from bronchial secretion After heavy medication with penicillin the patient improved transiently but then she became worse with detoriation and epileptiform seizures Increasing sputum blocked the air passage On treatment with Elkosin® (sulphaisodimidin) the chest roentgen picture became almost normal and nocardia disappeared from the trachea But the blood values decreased and finally she became unconscious and died

Autopsy In the lungs solid yellow foci and a system of cavities were noted The kidneys showed several yellow streaks and a larger necrotic focus which continued into a perirenal abscess with greenish thick necrotic material Such abscesses were also found in all parts of the brain and in musculature (neck and leg) Nocardia could be demonstrated in cultures with material from brain and kidney but in no other organ

The changes of the oral mucosa were the same as in earlier reported cases The polydysplastic case showed a peculiar discharge of well defined parts of connective tissue demarcated by ingrowing epithelial strands

In the tooth germs heavy disturbances in the enamel formation were found such as metaplasia of the enamel epithelium with many epithelial whorls In micro radiograms these appeared as globular irregularly mineralized structures The enamel was considerably thinner than normal In the case where the teeth had erupted they showed severe hypoplasias similar to those clinically seen in amelo genesis imperfecta In all the cases the tooth germs showed the same disturbances It is concluded that amelogenesis imperfecta may form a stigma in the reported forms of epidermolysis bullosa hereditaria

18 *B Larsson* ON MALIGNANT LIVER AND BILE DUCT TUMOURS IN CASES EXPOSED TO THOROTRAST AND AMONG WINEGROWERS WITH LONG STANDING EXPOSITION TO ARSENIC

The two groups weere briefly presented as available in the litterature The non epithelial tumours were mainly discussed carcinomas being further referred to elsewhere Some differences between the radioactive ThO_2 and the chemically toxic As_2O_3 were mentioned The Th sarcomas appeared after an average time of 16 years and the As tumours after 20 years The Tumour Development Time (TDT) in these human series represents Tomour Deaht Time in experimental series a Tumour Detectable Time might be registered

It is obvious that different substances can induce similar tissue responses In the present *two* sarcoma groups there is *one* cellular main response and *one* terminal lesion

Human tumours possibly exogenously induced and certain characteristics in the response of some animal species to carcinogenic influence were considered to present a tentative basis for a further study of common denominators in the process of malignant development

19 *S Dahlgren* RENAL CELL CHANGES AFTER INFUSION OF LOW VISCOSITY DEXTRAN

During the last year a great number of cases with changes in the renal tubules of the type known as osmotic nephrosis have been found at post mortems at the Karolinska sjukhuset 31 cases featuring such changes have been diagnosed and out of these 27 had been given so calleed low viscosity dextran (average molecular weight 40000) in amounts ranging from 100 to 4400 cc The other four cases had been given rather large doses of various hexosis It has not been possible to determine whether the tubular changes are accompanied by disturbances of function In spite of the fact that the commonly used type of dextran (average molecular weight 75000) has been widely used only 2a cases with tubular changes have been reported *in man after* infusion of dextran of the latter type (*Johnston & Lundy* 1953 *Vicery* 1956 and *Zettergren* 1962) The great number of cases with histo-pathological changes in the renal tubules after infusion of low viscosity dextran seems striking

20 *S Sregr* ISOLATED TOXOPLASMA MYOCARDITIS IN A CHILD

Necropsy report of a 5 year old boy who was found dead in his bed The boy had often had infections of the respiratory tract During the previous 6 months he had been fairly depressed

implantation of tantal plates It is suggested that neuroglia may give a malignant response to a similar stimulus

14 *C Lundmarl* A FATAL CASE OF VARICELLA

15 *I Olding* EPIDERMOLYSIS BULLOSA LETHALIS SOME MORPHOLOGICAL OBSERVATIONS

Sections of the skin from four infants who all died within nine weeks after birth and who had all been affected with epidermolysis bullosa lethalis were examined by conventional histological methods Sections were stained with haematoxylin and eosin and Weigert s elastin stain The argyrophile reticulum fibers were visualized by Laidlaw s method Sections of skin from dead infants at different ages and without skin diseases were used as controls

The early *changes of epidermolysis bullosa lethalis were multiple intracellular* vesicles in the basal layer of the epidermis The subepidermal elastin plexus was practically absent but this was also the case in the controls This *coincides* with the observations of *Dick* (J Anat *81* 201 1947) who has shown that the subepidermal elastin plexus is practically absent in the normal skin in infancy *On the other hand the reticulum fibers in the uppermost dermis* were well developed both in the cases of *epidermolysis* bullosa lethalis and in the controls These reticulum fibers were prominent also at the margins of recent bullae where the basal layer showed a marked vesicular degeneration

16 *B Robertson* REPARATIVE PHENOMENA IN HYALINE MEMBRANE DISEASE

In an autopsy material of 117 neonatal deaths varying in age between 6 hours and 6 days all displaying pulmonary hyaline membranes proliferative and reparative *phenomena in the alveolar epithelium were observed in cases surviving more* than two days This agrees with the observations recently reported by *Boss & Craig* (Pediatrics 29 890 1962) Similar changes in the alveolar epithelium were also observed in four other autopsy cases prematures varying in age between 10 and 21 days The clinical picture in these four cases had been that of idiopathic respiratory *distress since shortly after birth Histological sections from the lungs* displayed thickened alveolar walls containing fibroblast like cells and excess of collagen and reticulin No hyaline membranes of the usual type were observed in these sections although small amounts of hyaline material were present in some alveoli

These findings reflect a process of repair which indicates previous damage to the alveolar walls possibly *connected with the development* of hyaline membranes But since the author has also observed similar changes in the alveolar walls of 2 3 week old neonates without typical clinical history of idiopathic respiratory distress it is too early to draw any definite conclusions A clinical analysis of these cases is in progress

17 *T Arnell A Bergenholt* and *O Olsson* EPIDERMOLYSIS BULLOSA HEREDITARIA A HISTOPATHOLOGICAL STUDY OF TEETH AND ORAL MUCOSA IN THREE CASES

Demineralized sections and microradiographs in ground sections from the jaws of patients with polydysplastic and letalis forms of e b h were studied

FROM THE DEPARTMENT OF PATHOLOGY SECTION II UNIVERSITY OF HELSINKI HEAD PROFESSOR HARALD TEIR M D

OBSERVATIONS ON EFFECTS OF ILEUM RESECTION ON GRANULOCYTE COUNTS IN BLOOD IN RAT[1]

By

HARALD TEIR, TAPIO RYTOMAA and ANTTI CEDERBERG

Received 2 i 63

It has been possible to show that granulocyte production is enourmous even in normal conditions, *e g* in man c 15×10^{10} per diem (*Craddock* 1962) Since in a steady state the elimination must be equal to production, the question that arises is where such a mass of cells is eliminated

Little is known about the elimination of granulocytes However, from our present knowledge it can be regarded as obvious that leukocyte elimination occurs chiefly extravascularly (*Lissac* 1957, *Bierman* 1961, *Craddock* 1962) No quantitatively significant number of disintegration forms of granulocytes has been found in the bone marrow and blood in normal conditions (*Undritz* 1941, *Gross* 1954a, *Petrakis et al* 1957) Information on the life span of granulocytes and on their blood stream phase (*Osgood* 1954, *Lissac* 1957, *Craddock* 1962), on the ratios of the total granulocyte counts in different organs (*Osgood* 1954, *Donohue et al* 1958 *Rytomaa* 1960), and on other kinetics of granulocytes (*Craddock et al* 1959, *Finch & Hollongsworth* 1959 ...

... of several investigators (*Gross* 1954b, *Mashoff* 1958 *Rytomaa* 1960) is that there are surprisingly few disintegration forms of granulocytes in extramedullary tissues especially in view of the enourmous number of cells that is produced This makes it improbable that the disintegration of granulocyte ... could be the main phen...

It seems from the rec...

... 1955, 1959) who in several

[1] Sponsored by *the Damon Runyon Memorial Fund* (DRG 291 F and 644) New York and by the *Sigrid Juselius Foundation* Helsinki

The only gross finding of interest at autopsy was enlargement of the heart (wt 120 gm) Microscopic examination, however, revealed focal myocarditis with accumulations of lymphocytes around necrotic muscle fibres An intracellular, thin-walled pseudocyst, packed with crescent-shaped or round parasites about 3-4 microns long and with distinct nuclei rich in chromation was also seen Its appearance was that of a Toxoplasma pseudocyst No inflammatory reaction was seen around the pseudocyst The groups of lymphocytes in the myocardium probably marked the sites of ruptured pseudocysts

Toxoplasma-myocarditis occurs in both an acute and a chronic form The prognosis of the latter is much better than that of the former. Cases of myocarditis of unknown origin may be due to Toxoplasma It is, however, difficult to distinguish between Encephalitozoon, Sarcocystis and Toxoplasma

Resection of the large intestine was performed similarly by first ligating the ileum a distance of a few cm from the ileocaecal region The resected portion contained ca 50 per cent of the large intestine

Before the abdominal cavity was closed it was cleansed carefully by physiological saline irrigation in order to remove coagula of blood The irrigation was completed by leaving ca 5 cc of the solution in the abd minal cavity of the animal All the animals were given 2000 units of procaine penicillin intraperitoneally

Splenectomy and nephrectomy The former was done thr ugh a short upper median incision and the latter through a left flank incision by performing simple ligation of the vessels in the hilar region Hist logical samples were taken from the

tract was lifted out through the wound and the abdominal cavity was cleansed by physiological saline irrigation Surgery was concluded by giving all the rats 2000 units of procaine penicillin intraperitoneally

The blood samples—2 3 times preoperatively—were always taken before noon from the tip of the tail The leukocyte counts were performed in the customary way in Burker s counting chamber and for the eosinophil counts the samples were stained with phloxine in propylene glycol and counted in Fuchs Rosenthal s counting chamber The smears for the differential counts were stained after air drying with May Grunwald Giemsa All the animals of some test and contr l gro s were

s

counted in a counting chamber (Bu
stained in the usual way with M
fixed in 10 per cent neutral for
haemalaum eosin and benzidine
from tissue specimens and bone r

The final material of 80 rats

Group 1—the animals which lived ... more than a week

	Group 1	Group 2	Total
Ileum resection	12	6	18
Colon resection	5	3	8
Nephrectomy	2	2	2
Splenectomy	10	–	10
Sham operations	29	11	40
	58	22	80

Rat leukocyte values have been stated ...
t 30 000 per cu
been found to be
Since a decrease
be regarded as a
geometric mean
cyte counts ... the blood leukocyte and granulo

For the statistical analysis the results were checked by Wilcoxon s non parametric method[7]

nes

investigations, using isotope labelling techniques, point to the possible rôle of the lungs as the principal site of granulocyte elimination. According to Bierman, some other routes, e.g., the gastrointestinal canal, urine, and saliva may also play a certain rôle in the physiological elimination of leukocytes. *Pilzurra & Frascarelli* (1948) have assumed that the gastrointestinal canal is the main route of leukocyte elimination. In a more recent study, *Ambrus & Ambrus* (1959) reported that at least labelled lymphocytes were eliminated in the intestinal tract through mucosal diapedesis.

In his review of the "life span" of leukocytes, *Lissac* (1957) concludes that there is little evidence about the routes of leukocyte elimination and that, though the lungs seem to play the most important rôle, they can with reasonable certainty be said only to function as a reservoir of leukocytes. *Bierman* (1961), on the other hand, concludes in his review of homeostasis of leukocytes that "it is apparent that the major site of leukocyte destruction is probably extracirculatory, perhaps in the reticulo-endothelial tissues of the lung, spleen, liver, etc"

Several authors have established that the extravascular "reservoirs of white cells are not easily mobilized (*Osgood* 1954, *Craddock* 1960). This fact and the observation made in our laboratory, that the gastrointestinal tract contained a very large part of the extramedullary eosinophils in rat (*Rytomaa* 1960), directed our attention to this organ as an important site of granulocyte elimination.

In the present investigation the effect of ileum resection on granulocyte counts in blood has been studied. Though it is clear that this is far from an ideal method to investigate granulocyte elimination, certain information, however, seems quite possible. It should be mentioned in this connection that in reports of the effects of intestinal resections in human, changes in kinetics of granulocytes have not received any special attention.

MATERIAL AND METHODS

A total of 109 white male rats of Sprague Dawley strain aged c. 5–6 months were used as test animals. The blood picture of rat can be considered to have reached its final normal level at this age (*Rytomaa* 1960).

The actual method used was ileum resection. Colon resection, splenectomy, nephrectomy and sham operation were selected as control operations. Food was withheld from the rats used for the experiments on the evening preceding the operation. It was allowed again from the last postoperative day. The rats were given water *ad libitum* throughout. Blood samples were taken from all the animals on the morning of the operation day; the operations were performed in the afternoon under ether narcosis. After the operations the rats were moved in groups of 2–3 into clean cages.

Intestinal resections. Laparotomy was done through a fairly long midline incision. Approximately 5 cm from the ileocaecal region the ileum was closed with a silk ligature, care being taken not to injure the delicate mesenterial veins. A piece 25–60 cm in length was then resected from the small intestine. The total length of the small intestine in the rats used was ca. 80 cm. Samples were taken from the resected small intestine for histological preparations. Side to side anastomosis was performed using continuous sutures—at first in one layer, later after improvement of the operative technique in two layers.

TABLE 1

The Number of Leukocytes and Neutrophils in the Peripheral Blood Before and After Operation, by Experimental Groups

Group	Before operation			1-3 days after operation			4-8 days after operation			9-14 days after operation		
	No of rats	Leukocytes per mm³*	Neutrophils per mm³*	No of rats	Leukocytes per mm³*	Neutrophils per mm³*	No of rats	Leukocytes per mm³*	Neutrophils per mm³	No of rats	Leukocytes per mm³*	Neutrophils per mm³*
Sham operation	40	14 000	2 480	40	15 800	4 380	29	12 600	2 530	29	17 100	3 170
Splenectomy	10	17 200		10	21 100		10	31 000		10	25 800	
Nephrectomy	4	10 700	2 240	4	18 600	(3 910)§	2	19 800	(3 700)§	2	(14 800)§	(3 140)§
Colon resection	8	13 900	1 920	8	14 200	5 080	5	14 200	3 900	4	16 500	3 800
Ileum resection	18	16 300	2 460	18	11 300	3 690	12	18 500	4 100	11	19 300	4 950

* Geometric mean
§ Geometric mean of two samples

RESULTS

Neutrophil and Leukocyte Counts in Peripheral Blood

Ileum resection Distinct leukopenia was demonstrable during the first 3 days in the animals on which ileum resection was performed (Table 1 and Figs 1 and 2) Compared with the preoperative leukocyte values the difference was statistically highly significant ($P < 0\ 001$) Compared with the rats treated with colon resection and other control operations, the leukocyte counts of these animals were again lower ($P < 0\ 001$)

The absolute neutrophil counts calculated from the differential counts were distinctly lower in the rats with resected ileum than in the other animal groups ($P < 0\ 001$) The difference was smallest compared with the resected colon animals, but again it was statistically significant ($P < 0\ 01$) (Fig 2) Compared with the initial values, however, the neutrophil amounts established 1–3 days postoperatively were slightly elevated also in the animals treated with ileum resection (Table 1)

In the rats treated with ileum resection whose blood picture was followed at intervals of ca 12 hours for the first 3 days, all the changes reported above were demonstrable in the first sample taken after 12 hours The number of neutrophils, however, was slightly higher in this phase than later

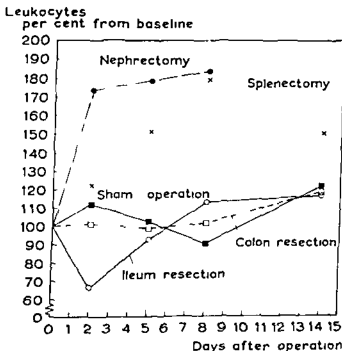


Fig 1

Per cent changes of leukocyte counts in peripheral blood after ileum and colon resections splenectomy nephrectomy and sham operation Preoperative cell counts as baseline

the first 3 days did not decline compared with the sham operation groups and with the preoperative values (Figs 1 and 2) The transient leukocytosis and leukopenia established in the animals with resected ileum were not demonstrated in these rats

Nephrectomy and splenectomy Pronounced leukocytosis was established in the nephrectomised and the splenectomised animals during the observation time (Table 1 and Fig 1)

Sham operations Manifest neutrophilia was observed in these rats during the first 3 days, but only relatively slight leukocytosis (Table 1 and Fig 1) Later on the leukocyte values remained relatively even and no leukopenia or transient leukocytosis was demonstrable.

Eosinophil Counts in Peripheral Blood

As expected, blood eosinopenia was established in the first few postoperative days in all the animals undergoing surgery (Table 2)

New, "spontaneous" eosinopenia was demonstrable in *all of the* groups on approximately the 7th day In the majority of the animals this eosinopenia was more pronounced than that established immediately (Table 2)

The eosinophil counts of the splenectomised animals were slightly higher than the initial values except during the eosinopenias seen in the first few days and on the 7th postoperative day (Table 2)

TABLE 2

The Number of Eosinophils in the Peripheral Blood before and after Operation by Experimental Groups§

Group	Before operation		1-3 days after operation		5 days after operation		7 days after operation		9-11 days after operation	
	No of rats	Eosinophils per mm³*	No of rats	Eosinophils per mm³*	No of rats	Eosinophils per mm³*	No of rats	Eosinophils per mm³*	No of rats	Eosinophils per mm³*
Sham operation	30	364	30	232	19	345	19	266	19	316
Splenectomy	10	463	10	356	10	817	10	267	10	505
Colon resection	6	275	6	137	3		3	71	3	375
Ileum resection	15	450	15	155	9	188	9	104	8	180

* Arithmetic mean

§ Nephrectomy rats are not included because there were only 2 samples for each

Total Cell Counts in Bone Marrow

The changes demonstrated in the bone marrow are reported very briefly in the present investigation

The total cell count in the bone marrow was distinctly higher on the 3rd than *on the* 2nd postoperative day in the animals with resected ileum The type of change was similar in those with resected colon, but the total cell counts were even below control values (Fig 3)

The leukocyte values of individual animals began to show considerable variation after the slight increase in the number of leukocytes established around the 9–10th day. The increases and decreases seemed to happen independently and did not follow any demonstrable rhythm.

Transient (duration shorter than 1–2 days), very pronounced leukocytosis was demonstrable at a later phase in two-thirds (8/12) of the animals with resected ileum. The leukocyte counts generally rose to 50 000–60 000 per mm³, the highest value established was 76 750. Concurrent eosinophilia and absence of the shift to the left in the differential count was observed during these leukocytosis attacks. The general good condition of the rats was unchanged during the leukocytosis attacks. Rats with obvious infection (not included to the final material) showed distinctly different changes in the blood picture, i.e., eosinopenia and shift to the left in the differential count. Furthermore, the leukocytosis in rats with obvious infection was much less pronounced than in rats with "transient leukocytosis".

Two thirds (7/12) of the rats surviving for longer period developed pronounced, acute leukopenia (not preceded by leukocytosis), sometimes with nearly total agranulocytosis, during which they died rapidly.

Colon resection. In the animals with resected colon, on contrast to those with resected ileum, the leukocyte and neutrophil values during

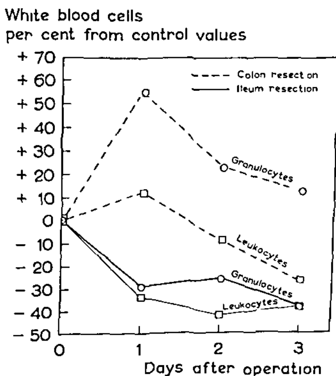


Fig. 2.

Per cent changes of neutrophil and leukocyte counts in peripheral blood during the first 3 days after ileum and colon resections. Cell counts of sham operated animals as baseline.

TABLE 3

The Number of Erythrocytes in the Peripheral Blood after Operation some of the Experimental Groups

Group	Under two weeks after operation		Over two weeks after operation	
	No of rats	Erythrocytes (in millions) per mm³*	No of rats	Erythrocytes (in millions) per mm³*
Sham operation	6	6.8	6	6.9
Colon resection	5	7.0	5	6.5
Ileum resection	7	6.0	7	5.9

* Arithmetic mean

Weight of the Animals

The weight of the test animals fell sharply—ca 20 per cent—in the resected ileum group during the first 2 weeks, in the resected colon group less—ca 10 per cent—and in the sham operated group not at all. The weights of the resected ileum animals generally did not return to the initial value, not even at a later stage (Table 4)

TABLE 4

The Weights of the Animals before and after Operation some of the Experimental Groups

Group	Before operation		Under two weeks after operation		Over two weeks after operation	
	No of rats	Weight in grams*	No of rats	Weight in grams*	No of rats	Weight in grams*
Sham operation	6	240	6	240	6	250
Colon resection	5	235	5	210	5	220
Ileum resection	7	230	7	175	7	195

* Arithmetic mean

DISCUSSION

There are reports in the literature on the possible significance of the gastrointestinal canal *for the removal of leukocytes*. In numerous older studies with different animals granulocytes have been found in histological preparations in the gastrointestinal tract between the mucosal cells and sometimes even in the lumen (*Weil* 1920, *Maximow* 1927, *Hamperl* 1932). *Pilzurra & Frascarelli* (1943) examined 10 persons for the total cell quantities in the urine, saliva, gastric juice, bile, and faeces and from the values *obtained* in the counting chamber and smears calculated the number of leukocytes eliminated per 24 hours along these routes. The *results* showed that the gastrointestinal canal was probably the principal site of leukocyte elimination. Only very few

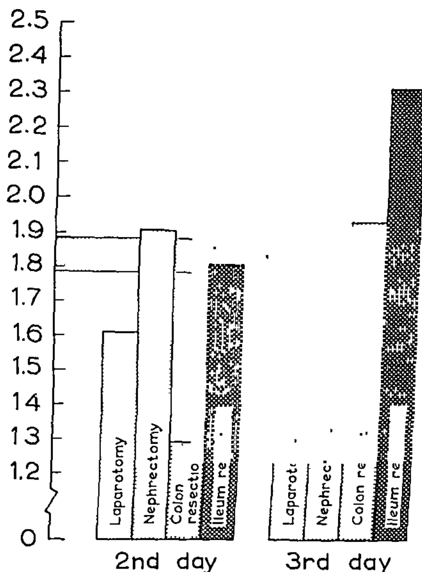


Fig 3
Total cell counts per mg of bone marrow on the 2nd and 3rd day after ileum and colon resections nephrectomy and sham operation Cell counts (range) of 4 untreated male rats of the same population as baseline

Erythrocyte Counts in Peripheral Blood

Throughout the observation period the erythrocyte values of the rats with resected ileum and colon did not differ significantly from values of animals given a sham operation (Table 3)

days) and the unchanged general good condition of the rats could be interpreted as phenomena against infection Furthermore the changes in blood picture in rats with obvious infection were quite different from these leukocytosis attacks Thus it seems possible that the causative mechanism could have been a disturbance in the elimination of granulocytes

Severe leukopenia which at times approached complete agranulocytosis was demonstrable upon the death of the animals and sometimes even at other times in rats surviving ileum resection for more than 2 weeks The diffenrential count often revealed a distinct shift to the left which can be regarded as an indication of the absence of a serviceable granulocyte reserve However the absence of a marrow granulocyte reserve was not necessarily due to exhaustion of granulocyte production since it was later observed in other series of experiments that the total number of myeloid cells in the bone marrow was not decreased The observation could be explained by a maturation arrest (malnutrition?) or alternatively by a compensatory inhibition of granulocyte maturation because of the block in peripheral elimination About two thirds of the rats died during the leukopenia of an obvious secondary infection

The results of the present investigation lend support to or at least do not disagree with the hypothesis that especially the small intestine has an important role in the elimination of granulocytes However according to the recent literature there seems to be some general agreement on the possible significance of the lungs in the removal of granulocytes (*Bierman et al* 1955 1959 *Fissac* 1957) It is obvious that granulocytes in relatively numerous amounts even can be found in the sputum but to the best of our knewledge the quantitative impor tance of this
be mentioned
cytes produce ... hours corres ponds in volume to ca 75 ml [if 1 ml is equivalent to 2 × 10⁹ granulocytes (*Tivey et al* 1951)]

According to the observations already referred to in the introduction it seems improbable that the disintegration of granulocytes in the bone marrow blood and in most extramedullary tissues could be the m...

... 1955) that the amount of granulocytes removed in 24 hours via the saliva and oral mucosa urine skin and serous membranes seems to be practically insignificant compared with their production

According to the available litterature it seems evident that granulocyte elimination takes place in extravascular tissues (*Fissac* 1957 *Bierman* 1961 *Craddock* 1962) It follows that either the granulocyte turn over is rapid in that organ which plays the most important role in

more recent investigations, using radioisotope labelling techniques, suggest the intestinal tract as a possible site of leukocyte elimination (*Bierman et al* 1955, *Ambrus & Ambrus* 1959, *Astaldi* 1960), and they do not cite any quantitative values *Ambrus & Ambrus* (1959), however, reported that at least labelled lymphocytes were eliminated in the intestinal tract through mucosal diapedesis

Our interest in the kinetics of granulocytes was aroused by investigations on eosinophil granulocytes (*Rytomaa* 1960) These studies established that in rat eosinophils are ca 200 times more numerous both in the bone marrow and the extramedullary tissues than in the blood Especially the very large proportion of extravascular eosinophils in the gastrointestinal tract in rat directed our attention to this organ as a possible principal site of granulocyte elimination

To test the validity of this assumption, we began by studying the effect of resection of the intestine on the granulocyte counts in the blood, bone marrow, and in various tissues It is clear that the method used in the present investigation may have caused many nonspecific effects As the quantity of granulocytes in peripheral blood in particular is influenced by numerous factors, some changes established concurrently in the cell counts of the bone marrow were considered briefly Furthermore, on the basis of our direct observations on granulocyte elimination in the intestinal tract it seems quite probable, that the results obtained in the present work warrant certain conclusions regarding the specific role of the *intestinal tract on the kinetics of granulocytes*

The investigation established that ileum resection leads to leukopenia *and, compared with the other operations, including colon resection*, neutropenia lasting for ca 3 days On the third day after ileum resection the bone marrow cell counts, by contrast, were increased to above the normal level although they had been *normal on the preceding* day The decreases in the total of cells in the bone marrow on the second day were obviously caused by the mobilization of the marrow granulocytic reserve (*Craddock et al* 1960), the increase *on the third* day probably by the stimulation of production Alternatively, the increase on the third day could be explained by an inhibition of granulocyte release from the bone marrow, probably because of a block in the peripheral elimination mechanism Similarly, the increase in leukocyte values in peripheral blood occurring ca 9 10 days after ileum resection suggests stimulation of *cell production in the bone marrow* or, alternatively, inhibition of cell elimination in the periphery

Pronounced attacks of leukocytosis of very short duration in which the number of cells generally increased to values *approaching leukemoid* reaction were established in two thirds of the rats undergoing ileum resection whose blood picture was followed at 1 2 day intervals *The concurrent* eosinophilia, *the absense of the shift to the left in the* differential count, the short duration of the reaction (shorter than 1–2

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granulocyte elimination, or the organ must be large Thus, bearing in mind the generally accepted view of the *defensive function of granulocytes*, it is not unlikely *a priori* that the intestine has an important rôle in the elimination of granulocytes

SUMMARY

Resections of the small and large intestine, splenectomy, nephrectomy, and sham operation were performed on 109 male rats in order to study the effect of these operations on the granulocyte counts in the blood The final material included 80 of these rats, since animals with obvious infection were excluded

A distinct leuko- and granulocytopenia in the peripheral blood was demonstrable during the first 3 days after the ileum resection In the other groups, the usual postoperative leuko- and granulocytosis occurred

In the rats with resected ileum the leukocyte values of individual animals showed considerable variations from the 9–10th day High, transient (under 1–2 days) leukocytosis approaching leukemoid values was established at a later phase in two-thirds of the animals The leukocyte counts generally rose up to 50 000–60 000 per cu mm No evidence of infection was noted in the blood picture, the general condition of the rats remained unchanged Two-thirds of the rats surviving for 3–4 weeks or more developed acute marked leukopenia—independent on leukocytosis attacks—during which they died rapidly Leukocytosis and leukopenia of this kind did not occur in the colon resection or other experimental groups A noteworthy leukocytosis was established in the nephrectomised and the splenectomised rats

A "spontaneous" eosinopenia on the 7th day was observed in all experimental groups in addition to the usual postoperative eosinopenia There was no change in erythrocyte counts

A preliminary study of the total cells counts in the bone marrow showed a distinct increase on the 3rd postoperative day in the rats with resected ileum

The present investigation lends support to, or at least does not disagree with, the hypothesis that the intestinal tract has an important rôle in the physiological elimination of granulocytes

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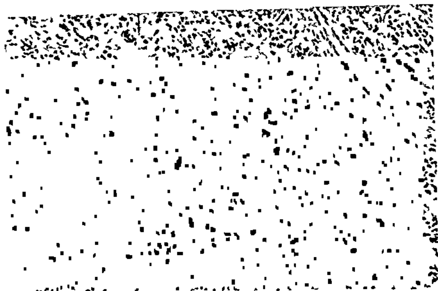


Fig 1

Biopsy taken 1941 from intumescence above the left orbit The diagnosis was Sympathicoblastoma H & F × 120

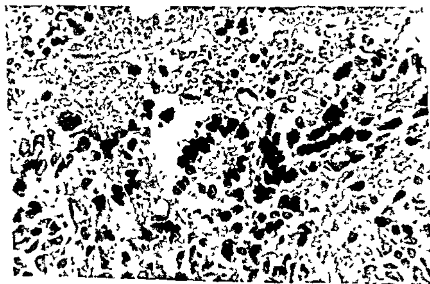


Fig 2

Higher magnificati n of a rosette from the slide shown in Fig 1 H & F × 384

FROM THE RADIUM CENTRE COPENHAGEN DENMARK
(HEAD PROFESSOR JENS NIELSEN, M D)
AND THE PATHOLOGICAL LABORATORY THE CENTRAL HOSPITAL RANDERS
DENMARK (HEAD DR J V THORBORG M D CHIEF PATHOLOGIST)

TRANSFORMATION OF SYMPATHICOBLASTOMA INTO GANGLIONEUROMA

With a Case Report

By

JAKOB VISFELDT

Received 10.1.63

In recent years clinical and experimental studies on embryonal tumours in children have appeared with increasing frequency, tumours apparently derived during the organogenesis from primarily non differentiated, multi potent cells Such cells have been assumed to retain their power of differentiation and it has been suggested that a differentiation might be therapeutically induced thus provoking a conversion into less immature benign tumours presenting reduced growth energy This theory, however, is based on clinical reports of a few cases only in which such conversion actually had been observed, mostly cases of sympathicoblastomata which, whether treated or not, converted into latent ganglioneuromata composed of apparently mature ganglion cells Only few of these reports, however, are based on histologically convincing data

The here discussed case has been described on two previous occasions, viz as case no 2 in a study from 1942 by *Thomas Rosendal* (12) and as case no 1 in a study from 1953 by *Poul Bjerre Hansen* (8) A metastasizing sympathicoblastoma originating assumedly from the adrenal cortex, had reverted in the course of intense radiotherapy and presented no signs of a further activity In 1962 the patient died from an intercurrent disease and was autopsied, it was realized that this was one of the rare cases in which a metastasizing sympathicoblastoma had "matured" into a highly differentiated ganglioneuroma Available histological data are published here together with a summary of the case history Theories concerning this type of tumour and clinical therapeutical endeavours are discussed

CASE REPORT

(Summed up on the basis of reports by *Rosendal* (12) and *Hansen* (8) together with the case record no 77944 from the Radium Centre in Copenhagen)

A b v born on February 25 1941 Disease set in in August 1941 the initial symp

directly distally to the adherance a pea sized perforation was noted from which a biopsy was taken The posterior wall probably also the pancreas were involved A biopsy was taken also from the posterior wall The histological examination of the biopsies showed a presence below the glandular tissue of a strange tissue rich in nuclei, much reminding of connective tissue formation with densely arranged irregular occasionally large and dark nuclei In the histological diagnosis the hypothesis was voiced that it might be tumour tissue

Hence the patient was referred to the Radium Centre in Copenhagen and on the not too well founded suspicion of recurrent tumour activity moderate doses of roentgen irradiation were given to the upper abdominal region totalling 1500 r to the anterior field 1450 r to the posterior field At his discharge from hospital the patient felt well

On June 26 1962 however haematemeses occurred and the patient was admitted to the medical department of the Central Hospital in Randers During his stay there haemorrhages were severe haematemeses as well as melaena assumedly attributable to an ulcer with ingrowth of tumour tissue The surgeons were consulted and it was deemed ill advised to institute surgical measures consequently the patient was treated by roentgen blood transfusion saline and glucose solutions Terminally the patient was running a high temperature and death occurred on July 16 1962

Autopsy performed by the author of these presents took place on the following day Data of the strange course of disease were sparse at this stage The gross findings included extreme paleness, some irregularity of the calvarial bones, slight protrusion of the eyes which, however, were not displaced in their sockets No lymph nodes were palpable

The pleural cavities on either side contained 1 liter a clear, slightly yellowish fluid and fibrinous membranes lined the pleural laminae The lungs presented stasis and oedema, dispersed minor haemorrhages, and numerous, delicate bronchopneumonias

Lining the trachea and below the bifurcation a number of enlarged lymph nodes were observed, the diameter of the largest being 3 cm A few lymph nodes were localized to the neck All were well-defined, in sections of a greyish, completely uniform, tough, and rather soft consistency

Gross inspection of the stomach and duodenum disclosed no tumour infiltration but distally by 1 5 cm to the pylorus a typical, 3 × 2 cm wide ulcer was demonstrated the bottom of which was of a greyish colour, necrotic, presenting a prominent process with a large vessel The ulcer invaded the entire wall, its bottom being in close apposition to the pancreas No tumour tissue was demonstrable by gross inspection of the ulcer Blood and melaena were abundant in the intestines

The liver and the bile ducts were natural, no metastases were observed, and also the pancreas was natural, the spleen weighed 250 g presenting the features of a typical, infectious spleen

The left suprarenal gland was natural In the retroperitoneal space, at the site of the right suprarenal gland, a large whitish tumour was demonstrable (Fig 3), it had no actual capsule but, being encircled by connective tissue, it was rather well-defined Its size was about 10 × 10 × 5 cm, the weight 250 g, the tumour was in close apposition to the uppermost, right, renal pole, its position being rather medial to the

toms including extravasation of blood in the eyelids frontotemporal swellings and some protrusion of both of the eyeballs The child was admitted to the paediatric department of the State Hospital (Rigshospitalet) where roentgen examination of the skull disclosed a radiating spicule formation in the frontotemporal intumescences Numerous small rarefactions were seen laterally in the frontal bone and in the right mandible Otherwise no skeletal anomalies were demonstrable The bone changes were interpreted—with certain reservations—as haemangiomata A large abdominal intumescence was palpable, extending from the right curvature as far down as to the right flank Roentgen examination of the abdomen showed at the site of the right kidney, a soft-tissue shadow, its size being as a half grape fruit numerous small calcifications were observed A biopsy was taken from the intumescence above the left orbit, and a histological diagnosis was established as sympathoblastoma (Figs 1 and 2) (G Teilum)

Roentgen irradiation was given to both temporal regions and to the tumour to the right in the abdomen, totalling 600 900, and 950 r to each field During and after treatment the general condition of the patient improved The intumescence above the orbits flattened the exophthalmus subsided, the abdominal tumour was found to have regressed a little on the right side but was now extending a little to the left of the first lumbar vertebra Roentgen irradiation was repeated a few months later with somewhat lower total dosages

In 1946 the patient was admitted to hospital again this time on account of some strange prominences developing in the skull particularly in the right parietal region where the cranial wall by roentgen examination was found to be very thin Sclerotic areas and some osteolytic spots were noted on either side in the orbital and temporal parts of the frontal bone, but no development of spicules

The prominences in the skull were exposed to serial roentgen irradiation the dosage totalling 1800 r to three fields The general condition of the patient was satisfactory but gradually his sight failed and a bilateral optic atrophy developed

In 1951 the patient was re admitted to hospital first to the neurosurgical department but later he was referred to the Radium Centre in Aarhus In all essentials the cranial bone changes remained stable, sclerosed areas with occasional rarefactions were observed around the orbits In addition isolated small sclerosed spots and rarefactions were seen in some of the ribs, in the left scapula in some of the vertebrae, and in the pelvis The tumour had displaced downwards and anterolaterally the right kidney, the left kidney was in normal position The symptoms were interpreted tentatively as tumour of the suprarenal gland with sclerosed

prevailed as regards the character of
onally might be rather tender, a total
from an occipital intumescence was
sue was found to be of a soft spongy

non characteristic nature histologically it was found to be composed of connective tissue with only few cells and vessels but symptoms were not demonstrable of active pathological processes (H Munck) Radiotherapy was discontinued

Development of the patient was satisfactory in the following years, he came under the care of the blind His intelligence seemed to be normal and his physical development was normal

In 1956 the left lobe of the thyroid gland was found to be the site of an adenomatid smooth swelling it could hardly be a metastasis from the sympathicoblastoma which remained completely inactive Extirpation however was found advisable and a leftsided hemistrumectomy was performed in Department K of the State Hospital The histological examination showed the presence of a cellular follicular adenoma of the thyroid gland (Hemming Poulsen)

During the following years the patient met for control at frequent intervals at the Radium Centre in Copenhagen in all essentials the condition seemed to be satisfactory

In January 1962 the patient felt diffuse abdominal pain and was admitted to the local hospital where the diagnosis acute abdomen was established On the day of admission the patient was operated a perforated gastric ulcer was sutured and appendectomy was performed

The gross appearance of the appendix was natural Behind the gallbladder displacing the latter to the fore and the kidney in a downward direction a retroperitoneal immovable rather bulgy tumour the size of a fist was found In an area of about 2 cm the anterior gastric wall adhered to the anterior abdominal wall

latter Consequently the right kidney had been slightly displaced in a downwards and lateral direction In the upward direction the tumour extended to the hilum of the liver without being connected with the bile ducts but closely adjoining the enlarged lumbar lymph nodes The entire tumour and the surrounding connective tissue were easily detached, they were completely independent of the gastro intestinal system Upon isolation of the tumour the right suprarenal gland was found to be embedded in the loose connective tissue encircling the tumour Gross inspection presented a normal suprarenal gland, there was no continuity between the suprarenal gland and the tumour In sections (Fig 4) the tumour and the lumbar lymph nodes presented a consistency of a rather uniform, whitish, rather whirled, solid, and tough character No soft areas were noted, nor haemorrhages and large calcifications

The weight of the left kidney was 140 g, of the right only 80 g, the latter being slightly deform The capsules were easily detached surfaces were smooth, and the parenchyma was normal

The spine was opened using a chisel and an about 1 cm large, greyish, rather soft focus of a metastatic aspect was found centrally in the body of the VIII thoracic vertebra The skull was opened and the brain excised Gross inspection disclosed no anomalies in the brain, cerebellum pituitary gland, pons, and medulla oblongata Also the cranial bones were opened with a chisel, *e g* both orbits, but no metastases were demonstrable The two optic nerves were dissected but pathological processes were not seen The prominence of the dorsum sellae was rather remarkable but normal bone tissue exclusively was seen when it was opened with a chisel

Specimens for microscopical examination were excised from various parts of the tumour, from the metastatic thoracic and lumbar lymph nodes, from both of the suprarenal glands, the lungs, spleen, and kidneys A small bone specimen from the dorsum sellae and one from the occipital bone were sent for decalcification but have been lost later

Slides Prepared Previously

Microscopy of biopsy taken in 1941 from the intumescence above the left orbit, labelled KP 1885 41[1]—Figs 1 and 2

The tumour [illegible] brils of the stroma [illegible] the tumour cells a[illegible] are immature, sph[illegible] with rather small, hyperchromatic nuclei and sparse cytoplasm some are larger, their nuclei more loose and the cytoplasm more abundant The tumour cells occur isolated or may be seen arranged in short chords, occasionally forming characteristic ro

[1] Professor *G Teilum* M D has kindly provided supplementary sections from the block for re examination

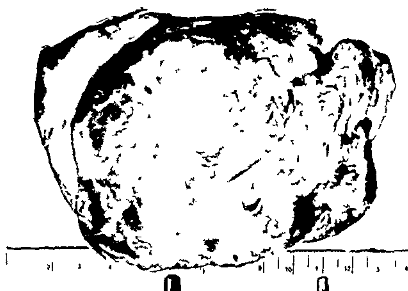


Fig 3

Tumour (ganglioneuroma) removed at autopsy 1962 from the retroperitoneal area above the right kidney

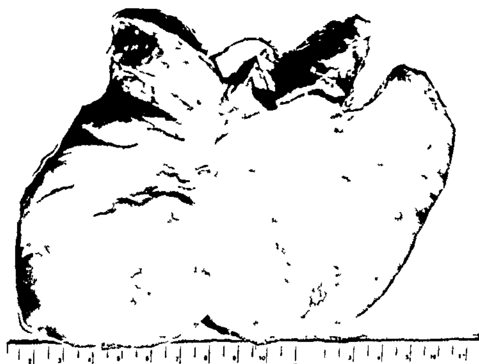


Fig 4

Cut surface of the tumour shown in Fig 3



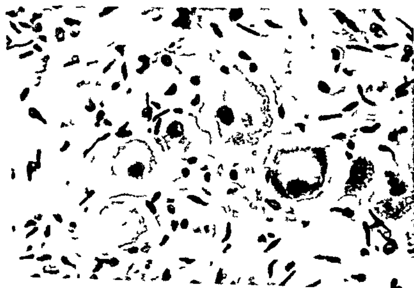
Fig 5
Section of the ganglioneuroma seen in Figs 3 and 4 Neurofibril staining × 1



Fig 6
Section of the ganglioneuroma shown in Figs 3 and 4 Area with collections of ganglion cells Gallocyanin chromalum × 300



F g 7
Sect on of metastat c thorac c lymph node removed at autopsy Gallo yan n chromalum × 181



F g 8
Deta l of F g 7 × 480



Fig 5

Section of the ganglioneuroma seen in Figs 3 and 4 Neurofibril staining × 120

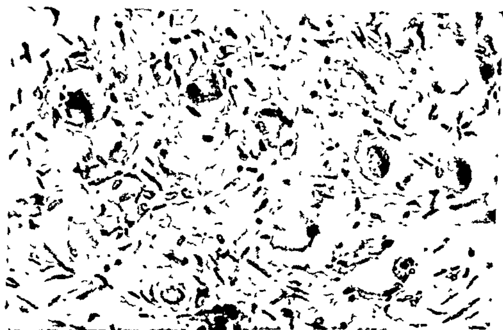


Fig 6

Section of the ganglioneuroma shown in Figs 3 and 4 Area with collections of ganglion cells Gallocyanin chromalum × 300

emergency hospitalization is required and, operation performed immediately upon admission, disclosed a perforated gastric ulcer On this occasion the large abdominal tumour is observed A biopsy is taken from the edge of the ulcer *Microscopy of the biopsy* suggests some tumour ingrowth into the stomach —At re-admission 5 months later on account of a bleeding ulcer, the institution of *surgical measures* is found inadvisable and the patient is *treated conservatively*, death occurs 3 weeks later Autopsy revealed the presence of a large duodenal ulcer, in the retroperitoneal space a well defined ganglioneuroma is found together with metastases of similar tissue to the lumbar, thoracic, and cervical lymph nodes, in addition a small focus is noted in the VIII thoracic vertebra, metastases to the skull are not demonstrable Diagnosis established on the basis of the histological examination of the tumour metastases to the lumbar and thoracic region present uniform ganglioneuroma tissue Areas presenting neuroblastoma tissue are not demonstrable

DISCUSSION

Neuroblastoma is the solid tumour most frequently found in infants and young children (5) Generally the tumours are diagnosed within the first year of life and may admittedly develop even prenatally (1) Most of the patients die very soon after birth but in rare cases the tumour has been seen to disappear or to "mature", *i e* the malignant cells convert into highly differentiated ganglion cells Hence, as observed by *e g* *Willis* (18) in embryologic studies, the parallelity of developments of tumours and normal ganglia seems to be very close The latter are derived probably from the neural crista and are found primarily as formations of densely arranged round cells, the cells may be arranged in parallel cords although the characteristic rosettes with the central delicate network of fibrils represent the most specific finding The mature ganglion cells and nerve fibres develop from these structures (18) Development of all of the ganglia in the autonomous system is not completed until the age of about 10 to 15 years has been attained up to this age the incidence of neuroblastomata has gradually abated Generally the histological features of neuroblastomata are characteristic, including the densely arranged round cells with formation of many rosettes or signs of the latter It should be expressly emphasized that although formation of rosettes represents a characteristic, morphologic feature, it need not always be demonstrable in the rapidly growing tumours and hence, it does not represent a necessary criterion on which to establish a diagnosis, these deliberations are in accordance with the above discussion of the development of ganglia

Cushing & Wolback (3) were the first who in 1927 reported a histologically verified case of malignant paravertebral sympathicoblastoma which had converted into a benign ganglioneuroma The follow up of

settes or their approximates No ganglion cells are observed The picture is characteristic of a *sympathicoblastoma*

Slides (labelled 587/62) of biopsies, taken in January 1962 on the occasion of the operation for perforated gastric ulcer, have been studied Re-examination of these slides failed to disclose a presence of tumour cells

Autopsy Slides

Tumour (Figs 5 and 6) The appearance is perfectly uniform in the various areas The tumour is composed of fibrillary tissue arranged in undulating strands and bundles Dispersed accumulations of isolated, highly differentiated and polymorphous ganglion cells are seen They may be ovoid or polygonal, and characteristic axons and dendrites are seen, many of the cells contain a brown pigment, in a few the nucleus is eccentrically arranged or the nucleus may be large and nodular, some present 2, 3, or 4 nuclei In the predominating, fibrillary tissue are some fibroblasts, but other cells may also remind of Schwann's cells Occasionally the nerve fibres are collected into circular structures as known from peripheral nerves Vascularization with delicate capillaries is abundant Numerous small calcified foci and some fatty infiltration are seen But tissue reminding of neuroblastoma is not demonstrable The picture is characteristic of a ganglioneuroma

Metastatic thoracic lymph nodes (below the bifurcation of the trachea, Figs 7 and 8) Features are similar to the ones described above The presence of some accumulations of lymphocytic cells suggests that it may be a matter of metastases to lymphatic tissue

The suprarenal glands Cadaverosis is vivid but otherwise structures are normal

Duodenal ulcer The presence of a typical, large, chronic, duodenal ulcer is demonstrated, lined on either side by Brunner's glands The ulcer invades the muscles, a small zone of fibrous tissue covering the underlying pancreatic tissue No tumour cells at all are demonstrable

EPICRISIS

A 20-year-old man is admitted to hospital with acute haematemesis

When the patient was 6 months old the presence of a large tumour in the right renal region had been ascertained metastasizing particularly to the skull A biopsy taken at that time from the intumescence above the left orbit suggested the presence of a sympathicoblastoma Intense roentgen irradiation to the abdomen and the skull had been administered upon which no further growth of the tumour occurred roentgen examination had verified the development of delicate calcifications in the tumour The cranial metastases retrograded An optic atrophy and reduced sight represented the sequelae Otherwise the development of the patient had progressed normally —In January 1962

whic [illegible] nal tumour originally had been demonstrated together with [illegible] patı [illegible] and the metastases in the skull 20 years later the patient dies from a bleeding duodenal ulcer, and the large tumour in the right renal region is demonstrated at autopsy It should be noted, however, that metastases were found also in e g the thorax to which no irradiation had been given In all of the specimens excised from the tumour and the metastases the presence of ganglioneuroma tissue is demonstrated exclusively, no neuroblastoma tissue

Therapy Various measures have been introduced in the treatment of neuroblastomata and the results obtained have given some reason to optimism These therapies, isolated or combined, include (20) 1) surgical, radical excision of the tumour, 2) roentgen irradiation, the therapeutical doses generally ranging from 1500 to 3000 r, 3) chemotherapies, previously using Coley's toxin, aminopterin, amino anfol, etc, lately particularly using B_{12} With a view to illustrating the results obtained the following studies shall be briefly referred *Wittenborg* (19) reported a material including 73 cases, 3 of the 28 non-treated patients (10 per cent) survived for 3 years or more 45 of the patients were treated by a) surgical measures, b) surgical measures combined with postoperative roentgen irradiation; or c) roentgen irradiation exclusively 30 per cent of the total survived for 3 years or more In 60 per cent of the cases "radical" extirpation of the local tumour resulted in survivals for 3 years, 60 per cent of the cases in which extirpation had not been radical but roentgen irradiation had been given, survived for 3 years *Phillips* (11) published a material collected from several hospitals totalling 155 cases, 22 6 per cent presented a 3 year survival or above *Horn et al* (9) reported 44 cases, 41 of the patients, follow-ups covering 14 months, included 16 patients who survived and became symptomfree and 25 patients who died These authors advocate the institution of surgical measures as radical as possible *Uhlmann & von Essen* (17) report a material including 20 patients, all of these were treated by roentgen irradiation In 7 cases the time of survival ranged from 22 months up to 13 years In all 7 cases the administration of roentgen had been based on biopsies In 5 of these cases metastases had been present at the time of treatment Reports are available, as already mentioned, of a few patients who had received Coley's toxin, probably without benefit (3, 4, 16) Recent studies by *Bodian et al* (2) are of much greater interest Here the results from administrations of large, long term doses of B_{12} are reported although it must be admitted that the patients had received additional treatment by conservative measures The material includes 72 children who all received the drug, 7 of these were followed for less than one year Regression of the tumour was seen in 28 of the cases—2? of these children are still alive and, with one exception, have been followed for one year or more, 2

the patient had covered 16 years. This case has been repeatedly discussed; the patient has been re-examined and the case has been reported again (together with another case) *e.g.* by *Fox et al.* in 1959 (4). Reports of several other cases are available (*e.g.* 10, 11, 16, and 17) but few of these are substantiated by reliable histological data. Reports are abundant, however, of cases in which regression is total.

In the course of regression or conversion of neuroblastomata into ganglioneuromata, whether spontaneous or during treatment, the initial symptoms generally include: haemorrhages, necroses, and small calcifications. Occasionally this process may be verified by roentgen examinations. Changes in the tumours need not be uniform and, according to experience, the appearance of the tumours, macroscopical as well as microscopical, may be highly different in the various areas including also the intermediate stages. The histological diagnoses most commonly established: neuroblastoma—ganglioneuroblastoma-ganglioneuroma—give evidence hereof. The theory that ganglioneuromata always develop from neuroblastomata seems obvious (14, 15). It has been mentioned already that neuroblastomata occur mostly in children although a few reports are available of developments later in life, *Halpert et al.* (7) e.g. found a metastasizing neuroblastoma in a 53-year-old patient, and *Willis* (18) describes a neuroblastoma developing in a 51-year-old patient. The explanation is probably that neuroblastoma elements in a ganglioneuroblastoma for unknown reasons have become stimulated to renewed growth and metastasization. According to expectation, ganglioneuromata become manifest later in life than neuroblastomata. Some are prone to give symptoms in childhood but many remain unnoticed until adult age and may not be diagnosed until the patient appears for a routine roentgen examination. Reports are available of cases even in which such tumours represented casual autopsy findings in middleaged or old patients (18). In fully differentiated, benign ganglioneuromata nothing but mature nervecells and nervefibres are demonstrable. Shapes of the cells may be ovoid, polygonal, or pyramidal like normal or degenerated ganglion cells. Not infrequently they contain a brownish pigment. The fibres are arranged in broad strands and bundles with or without Schwann's cells. Most, if not all, ganglioneuromata contain vast amounts of fibres with rather few and dispersed groups of ganglion cells. But so far it remains to be elucidated why this is so. In the rare cases of metastases or invasive growth from tumours histologically presenting the features of ganglioneuromatas the diagnosis, malignant ganglioneuroma has been suggested (*e.g. Uhlmann & von Essen*, case no. 18 (17). Still, mitoses are probably never present and the ganglion cells are of completely the same type as the ones encountered in the socalled benign ganglioneuroma. In my opinion these rare cases should be interpreted as originally metastasizing neuroblastomata the primary tumour of which together with the metastases have converted, a theory which is substantiated by findings from the here discussed case in

SUMMARY

In 1941 a 6 month old child was found to have a large, para vertebral tumour and multiple metastases particularly to the skull

of intense roentgen irradiation to the skull and the abdominal tumour the cranial metastases regressed and calcifications developed in the abdominal tumour The development of the patient progressed normally, but in 1962 he died from a bleeding duodenal ulcer At autopsy a well defined retroperitoneal tumour was found together with metastases containing similar tissue in the lumbar, thoracic, and cervical lymph nodes, in addition a small focus was noted in the VIII thoracic vertebra metastases to the skull were not encountered Histological examination of the tumour and the lumbar and thoracic metastases disclosed the presence of uniform ganglioneuroma tissue No neuroblastoma tissue at all was seen The question is discussed whether differentiation may be therapeutically induced in cases of neuroblastomata On the basis of previously reported studies the various therapeutical measures are discussed including operation irradiation, and chemotherapies Even widespread metastases do not contra indicate the application of therapeutical irradiation since regression or "maturing" of metastases occasionally may be obtainable even if they are not hit by the irradiation The here discussed case is in support of this contention

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of the children have died from intercurrent diseases For many reasons the results cannot be readily compared In general it may be concluded that operation always is indicated provided that it can be sufficiently radical, indeed it must be admitted that satisfactory results have been obtained also in cases in which the operation had not been radical but rather been combined with postoperative *roentgen irradiation, and results have been satisfactory* even in cases treated by roentgen exclusively The value of the B_{12} therapy remains to be elucidated In conformity with other investigators (17) some characteristic features should be emphasized it can hardly be denied that some of the tissue invaded by tumour cells has escaped irradiation in surviving, roentgen treated patients Hence, irradiation of certain areas of the tumours seems to provide regression or "maturing" even in some of the non-irradiated areas The case here discussed serves as example Widespread metastases do not represent a contra-indicant to intense radiotherapy

Being aware of the proneness of malignant embryonal tumours to differentiate and turn inactive, expectations have been great as regards the practicability of inducing therapeutically such *differentiation The problem* is fascinating although it should be borne in mind that, for all practical purposes, our insight into factors inducing spontaneous "maturing" is almost non-existent Hence, the therapeutical endeavours have been based on empiricism During roentgen irradiation neuroblastomata have been seen in rare cases to convert into ganglioneuromata but this finding should be taken with a certain reservation, always remembering that tumours are prone to spontaneous "maturing" Occasionally the roentgen irradiation may involve a direct cancerocidal effect (17) So far it remains to be elucidated whether or not such effect is involved also in the treatment using B_{12} If conversion into benign types occurs during treatment it would be rather unjust, on the basis of our present insight, to explain this feature as a direct effect of the therapeutical measures Until otherwise demonstrated it seems obvious that irradiation (possibly also chemotherapy) may exert a certain cancerocidal effect on the most immature of the tumour cells and hence allow the remaining, 'surviving" cells to pass through the process of differentiation which admittedly embryonal tumour cells do spontaneously The differentiation of embryonal tumours should be further examined, clinically as well as experimentally Neuroblastomata as well as ganglioneuromata may develop in animals (6, 13) and experiments using transplanted "mouse neuroblastomata" are in progress (2) *Goldstein & Pinkel* (5) professed to having demonstrated a differentiation into ganglionic cells using long term cultures with human neuroblastoma tissue *Uhlmann & von Essen* (7) emphasize the demand for histological follow-up studies of surviving patients — A further examination of the biological features of these tumours is required if ever we shall have hopes of arriving at a more effective therapy

FROM THE INSTITUTE OF GENERAL PATHOLOGY UNIVERSITY OF AARHUS AARHUS DENMARK (HEADS PROF J BICHEL AND PROF A STENDERUP)

THE THYROGLOBULIN POOL IN THE THYROID GLAND IN PATIENTS WITH AND WITHOUT THYROID AUTO ANTIBODIES

By

TAGE HJORT

Received 23 xii 62

During recent years numerous investigations have shown that auto immune reactions occur very frequently in a variety of thyroid dis orders (*Roitt & Doniach* 1958 1960 *Paine et al* 1957, *Blizzard et al* 1959 *Mackay & Perry* 1960) Three different types of thyroid specific auto antigens are now known The best studied of these is thyroglobulin (*Doniach & Roitt* 1957 *Witebsky et al* 1958), which normally occurs only in the colloid of the thyroid gland However the colloid also con tains another auto antigen—the second colloid antigen (CA 2)—the nature of which is as yet unknown While the thyroglobulin antibody can be demonstrated by precipitation the tanned cell technique and Coons technique CA 2 antibody can only be revealed by Coons tech nique (*Balfour et al* 1961) The third antigen is found within the cells in the highest concentrations in the glandular tissue of thyrotoxic pa tients (*Roitt & Doniach* 1960) The corresponding antibody is usually demonstrated by a complement fixation test (Complement fixing anti body) The cytotoxic effect produced by certain Hashimoto sera when these are added to fresh cultures of trypsinized thyroid cells has been ascribed to a fourth antigen antibody system (*Pulvertaft et al* 1959 *Irvine* 1960), but subsequent investigations suggest that the cytotoxic factor is identical with the complement fixing antibody (*Pulvertaft et al* 1961, *Irvine* 1962)

Immune reactions of the delayed hypersensitivity type may also occur Thus *Buchanan et al* (1958) showed that intracutaneous injec tion of human thyroid extract in certain patients with thyroid disease gives rise to cutaneous reactions similar to those of tuberculin The im portance of these immune reactions in man has not yet been clarified,

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C *Determination of the antigen content in the thyroid gland* —If the antibodies can be bound to the antigens in vivo, it might be expected that the antigen content in the thyroid tissue should be lower in patients with the thyroid antibody concerned than in those without this antibody

As the last approach to the problems has so far not been systematically utilized, this was employed in the present study

Of the three known thyroid auto antigens, *thyroglobulin* is easiest to determine Attempts were therefore made to obtain relative, but comparable measures of the thyroglobulin content in thyroid tissue, and the estimated concentrations were then related to the amount of antibody in the serum

MATERIALS AND METHODS

Patients —Thyroid tissue was studied from 71 patients who underwent operation on the thyroid gland Of the patients studied, 39 were thyrotoxic before operation Pre operatively they had been treated with iodine, and 13 had for periods, also received neomercazole or methylthiouracil A toxic adenoma was revealed in two of the toxic patients, while histological studies in the remaining 37 showed more [illegible] toxic tissue changes Thirty two goitre patients were euthyroid before

Demonstration of thyroid auto antibodies —The serum samples from the patients

Thyroglobulin antibody was demonstrated by *Boyden*s haemagglutination technique (1951) with formalin treated thyroglobulin coated sheep erythrocytes as previously described (*Hjort* & *Mogensen* 1961)

while the thyroglobulin antibody gave a

strated by *Donnelley*s semi micro method 1958)

Determination of the thyroglobulin content of thyroid tissue —At operation suitable tissue fragments were secured for histological examination and the remainder was at once stored at —20° C until the thyroglobulin determination could be performed Storage in this way for 1 2 months did not give rise to any change in the thyroglobulin concentrations measured The frozen thyroid tissue was divided in blocks of a suitable size (5 10 g), weighed and cut into thin slices and coarsely homogenized with saline in a Potter homogenizer The final volume was then adjusted to 10 ml per gramme of tissue The extracts were allowed to stand over night at 4° C, after which the sediment was removed by centrifugation In the majority of cases two extracts were made from each gland, usually one from each

but experiments with immunization of guinea pigs with homologous thyroglobulin have revealed a correlation between the occurrence of experimental thyroiditis and positive delayed skin thyroglobulin reaction, whereas no relationship was demonstrated between the development of thyroiditis in the animals and the presence of thyroglobulin antibody (*Miescher et al* 1961, *McMaster et al* 1961). *Felix-Davies & Waksman* (1961) were even able to transfer experimental thyroiditis in guinea pigs by means of lymphocytes. Simultaneously with the development of thyroiditis in the recipients, a positive cutaneous reaction to thyroglobulin occurred, whereas it was not possible to demonstrate any thyroglobulin antibody in the animals

When, for the first time, *Roitt et al* (1956) demonstrated a precipitating thyroid auto-antibody (thyroglobulin antibody) in the serum of patients with Hashimoto's disease, it was reasonable to assume that a causal relationship existed between the auto-immune reaction and the progressive destruction of the thyroid tissue which is characteristic of this disease Definite evidence in support of this assumption was produced by *Witebsky et al* who demonstrated that active immunization of rabbits with autologous thyroid extract (to which Freund's adjuvant was added) simultaneously with an auto-antibody production led to histological changes in the remaining thyroid tissue in the rabbits—changes, which were of essentially the same type as those seen in Hashimoto's disease (*Witebsky & Rose* 1956, *Rose & Witebsky* 1956) However, when it was realized that a number of different forms of thyroid auto-immune reactions exist, and that the auto-immune reactions may occur not only in Hashimoto's disease, but also in other thyroid disorders and even in persons without signs of such disease (*Hill* 1961), the problems involved have become fairly complicated At the present time, attempts must be made, as far as possible, to assess the importance of each of the various forms of thyroid auto-immunization separately In man, such an assessment may be performed on the following principles

A *Clinical evaluation* —Clinical observations have given reasons to believe that severe cases of auto-immunization will often end in myxoedema This has been confirmed by several recent investigations, which have concordantly shown that postoperative myxoedema mainly occurs among patients with complement-fixing antibody in the serum at the time of operation (*Gammie et al* 1961, *Buchanan et al* 1962, *Hjort & Mogensen* 1962, *Irvine et al* 1962)

B *Transfer of thyroid antibodies* —Nature herself performs this experiment by the passage of the antibodies through the placenta from the maternal to the foetal circulation Thus, large amounts of passively transferred thyroglobulin antibody and complement-fixing antibody have been demonstrated in newborn infants, apparently without having exerted any influence on thyroid function (*Blizzard et al* 1960, *Parker & Beierwaltes* 1961, *Hjort & Pedersen* 1962)

C *Determination of the antigen content in the thyroid gland*—If the antibodies c ight be expected that the ant be lower in patients with lose without this antib

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... previously described (*Hjort & Mogensen* 1961)

Antibody against second colloid antigen (CA 2 antibody) was demonstrated by *Coons' fluorescent test (sandwich technique) performed on methanol fixed frozen sections of thyroid tissue* (approx 6 μ) as described by *Balfour et al* (1961) Com-

positive serum of medium strength was included as a control As thyroglobulin antibody also stains the colloid only sera which had yielded a negative or very weakly positive reaction by the haemagglutination test were studied In the presence of CA 2 antibody uniform bright apple green staining of the colloid was observed —as described by *Balfour et al* (1961)—while the thyroglobulin antibody gave a floccular appearance of the colloid space

Complement fixing antibody was demonstrated by *Donnelley*'s semi micro method (1951) as described by *Roitt & Doniach* (1958)

Determination of the thyroglobulin content of thyroid tissue—At operation, suitable tissue fragments were secured for histological examination, and the remainder was at once stored at −20° C until the thyroglobulin determination could be performed Storage in this way for 1–2 months did not give rise to any change in the thyroglobulin concentrations measured The frozen thyroid tissue was divided in blocks of a suitable size (5–10 g), weighed and cut into thin slices and coarsely homogenized with saline in a Potter homogenizer The final volume was then adjusted to 10 ml per gramme of tissue The extracts were allowed to stand overnight at 4° C, after which the sediment was removed by centrifugation In the majority of cases, two extracts were made from each gland, usually one from each

TABLE 1

Immunological Determination of the Thyroglobulin Content in Thyroid Tissue.

Extract dilution		Dilution of test serum containing thyroglobulin antibody								Thyroglobulin concentration (µg ml)	Extracted thyroglobulin (mg g tissue)	
		1 100	1 500	1 2000	1 4000	1 8000	1 16000	1 32000	1 64000			
Extract 195	1 10 000	4	0	0	0	0	0	0	0	10	100	100
	1 20,000	4	3	0	0	0	0	0	0	5	100	
	1 40 000	4	4	1	0	0	0	0	0	>2	>80	
	1 80 000	4	4	4	0	0	0	0	0	>1	>80	
	1 160 000	4	4	4	4	0	0	0	0	>0 5	>80	
Extract 196	1 10,000	4	3	0	0	0	0	0	0	5	50	40 to 50
	1 20,000	4	4	1	0	0	0	0	0	>2	>40	
	1 40 000	4	4	4	1	0	0	0	0	1	40	
	1 80,000	4	4	4	4	2	0	0	0	0 5	40	
	1 160 000	4	4	4	4	4	0	0	0	>0.2	>32	
Thyroglobulin Standard	20 µg/ml	2	0	0	0	0	0	0	0			
	10	4	0	0	0	0	0	0	0			
	5	4	3	0	0	0	0	0	0			
	2	4	4	2	0	0	0	0	0			
	1	4	4	4	1	0	0	0	0			
	0 5	4	4	4	4	2	0	0	0			
	0 2	4	4	4	4	4	1	0	0			
	0 1	4	4	4	4	4	4	0	0			
	0	4	4	4	4	4	4	4	0			

4 = complete agglutination
3 = large ring
2 = medium-sized ring
1 = small ring
0 = no agglutination

lobe but in a few cases the amount of tissue was so small that only one extract

groups in the extracts
previously used for the
om the stock dilution of
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tion in
dilution

formalin treated, thyroglobulin coated erythrocytes was added ... sample
left overnight at room temperature and read next morning It appeared to be appropriate to distinguish between various grades of agglutination 4 was used for complete agglutination (i e sedimentation like a carpet), 3 for a large ring 2 for a medium sized ring 1 for a small ring and 0 for a negative reaction

A comparison of the haemagglutination inhibitions produced in the various
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absolute values However, *as the same standard and the same thyroglobulin antibody-containing test serum were used in all the determinations the values will be comparable* which was the only object in view

TABLE 2

The Influence of the Reaction Time on Immunological Determination of Thyroglobulin

Extract	Reaction time (in hours) at 37°C				
	1 hour	2 hours	4 hours	8 hours	24 hours
1	180	180	180	180	180
2	≤ 80	60	80	80	80
3	40	> 40	60	60	60
4	80	160	180	180	180
5	30–40	40	40	40	40

The figures *indicate* the amounts of thyroglobulin in mg/g tissue

Although the procedure is very similar to that used for the determination of thyreglchulin in serum the influence of certain factors must neverthel ... n
sidered in some detail

before the thyroglobulin coated erythrocytes were added The results (Table 2) showed that the antigen antibody reaction seemed to have been completed at 4 hours in all cases (As early as at 1 hour the standard exhibited the very constant inhibition pattern, as appears from Table 1)

2 *Influence of dilution on the extraction of thyroglobulin* —In two cases homogenized saline extracts were prepared in the dilutions 1 2 1 10 1 20 and 1 40 As the thyroglobulin determinations gave the same results on all extracts from the same gland the dilution normally used i e 1 10 must with great certainty result in a fairly complete extraction of the thyroglobulin

3 *Destruction of thyroglobulin in the extracts* —It is conceivable that when the extracts are allowed to stand overnight at 4° C, this might result in a certain decomposition of the thyroglobulin caused by co extracted enzymes In order to throw light on this problem, a large amount of extract was prepared in a cooled mixer (Ato Mix) from each of two goitres By this procedure which requires relatively large quantities of thyroid tissue complete homogenization is obtained Each of the two extracts were distributed in six tubes One sample of each extract was centrifuged at once and the supernatant stored at —20° C The remaining tubes were left at 4° C On each of the next five days one sample of each extract was centrifuged and the supernatant stored at —20° C Simultaneous thyroglobulin determinations were then performed on all the samples The results (Table 3) show that no destruction of the thyroglobulin in the extracts could be disclosed after standing at 4° C

TABLE 3

The Results of Immunological Determination of Thyroglobulin after Standing of the Extracts at 4° C for Various Periods

Time of standing (in 24 hour periods) at 4° C					
0	1	2	3	4	5
160	160	160	180	160	180
≧ 200	200	200	200	200	200

The figures indicate the amounts of thyroglobulin in mg/g tissue

TABLE 4

Thyroglobulin Content in Different Extracts from each of Three Goitres

Type of goitre		
Non toxic nodular	Toxic	Toxic
100 125	110	150 200
100 125	110	200
125	110	200
125	100–125	200
125	125	200
	125	200 250
	125	
	125	
	125 150	
	130	
	160	

The figures indicate the amounts of thyroglobulin in mg/g tissue

4 *Uniformity of the thyroid tissue* —In order to assess to what extent the result obtained in the study of 5 10 g tissue can be taken as representative of the thyroid gland concerned a series of extracts from different parts of the same goitre were studied in three cases The results are shown in Table 4 As the technique used in the present study merely aims at obtaining comparable levels of the thyroglobulin

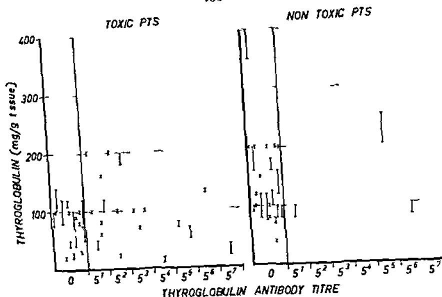


Fig 1
Thyroglobulin concentrations in thyroid tissue related to the amounts of *thyroglobulin antibody in the serum*

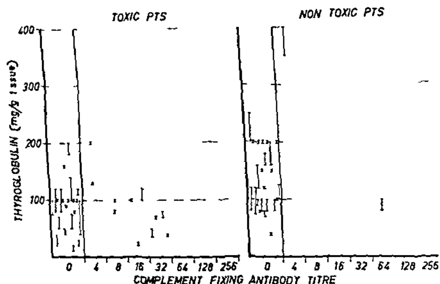


Fig 2
Thyroglobulin concentrations in thyroid tissue related to the amounts of complement fixing antibody in the serum

concentration in different glands, the agreement between the various results obtained in all three cases must be regarded as fully satisfactory. It also appears from Figs. 1 and 2 that fairly good agreement between the results for the two different extracts which were routinely prepared from each gland was generally obtained

In electrophoretic analysis, thyroglobulin migrates as an α globulin. Thyroglobulin will generally constitute the greater part of the α-globulin in the extracts, and at any rate, the concentration of thyroglobulin in the tissue will not exceed that of α globulin. Attempts were therefore made to obtain a control of the immunological thyroglobulin determinations by estimating the amount of α globulin in some of the extracts. The protein concentrations in the extracts were determined by a biuret reaction. Paper electrophoresis was performed with an LKB apparatus according to the lines suggested by the manufacturer, *i.e.* Schleicher & Schull 2043 B paper and a barbital buffer (ionic strength 0.125, pH 8.6) were employed. The electrophoresis was run for about 18 hours with a potential gradient of 2 V/cm. The strips were stained with bromphenol blue and read in an Elphor photometer.

TABLE 5
Antibodies Found in the 71 Patients Studied

Histological diagnosis	No. of patients	TGA + CFA	CA 2 A + CFA	TGA	CA 2 A	CFA	No anti-bodies
Diffuse toxic changes	37	8	0	12	5	3	9
Toxic adenoma	2	0	0	1	0	0	1
Non toxic colloid goitre (diffuse or nodular)	27	1	1	2	2	0	21
Non toxic adenoma	5	1	0	0	0	0	4

TGA thyroglobulin antibody   CA 2-A, CA 2 antibody
CFA complement-fixing antibody

RESULTS

The thyroid auto-antibodies revealed in the sera from the 71 patients are shown in Table 5. In nearly all the patients who had thyroid antibodies in the serum (including most of those with CA-2-antibody only), the histological examination revealed lymphocytic infiltration in the thyroid tissue. The presence of lymphocytic infiltration may, by displacement of the glandular tissue, give rise to misinterpretation of the thyroglobulin concentration in the active thyroid tissue. Only in two patients, *viz.* the only one with non-toxic goitre who had both thyroglobulin antibody and complement-fixing antibody in the serum and the toxic patient who had the highest thyroglobulin-antibody titre, were the lymphocytic infiltrations in the thyroid tissue so pronounced that the actual glandular tissue had been displaced. In the other cases with lymphocytic infiltration, the infiltrates constituted such a small fraction of the tissue that this cannot have exerted any appreciable influence on the results obtained.

In Fig. 1 the thyroglobulin levels revealed in the 37 toxic and 27 non-toxic glands with diffuse changes are related to the amounts of thyroglobulin antibody in the serum. It appears that the values for both groups are scattered over a wide range. On the whole, the thyroglobulin content was higher in the non-toxic than in the toxic glands. In another connection, *Wilebsky et al.* (1957) made similar observations. On the basis of the present investigation it cannot be said if the lower average level in toxic patients is solely referable to the disease, or if the pre-

operative treatment also plays a part The results in Fig 1 do not suggest any immediate relationship between the amounts of free, thyroglobulin antibody-fixing groups in the thyroid gland and the occurrence of thyroglobulin antibody in the serum There are thus no signs suggesting that the circulating thyroglobulin antibody in vivo is capable of neutralizing the antigen in the colloid follicles in the thyroid gland

In Fig 2, the thyroglobulin concentrations in the glandular tissue are related to the amounts of complement fixing antibody in the serum Among the thyrotoxic patients who had complement-fixing antibody it seems as if the thyroglobulin concentrations decreased with increasing antibody titres, but taken collectively, the results obtained in the patients with antibody do not differ from the observations made in those without antibody

In two of the eight patients in whom CA-2 antibody was demon-
str ' in the glandular
tis in the remaining
si e

In the seven cases in which the determinations were performed on tissue from solitary adenomata, relatively low thyroglobulin concentrations were observed The results are shown in Table 6

TABLE 6

Thyroglobulin Concentrations in the Seven Adenomata Studied

Histological diagnosis	Antibody titre		Thyroglobulin concentration (mg g tissue)
	TGA	CFA	
Toxic adenoma	25	0	20
do	0	0	50
Non toxic adenoma	625	4	35–70
do	0	0	80–100
do	0	0	10–15
do	0	0	25
do	0	0	80

In Fig 3, the concentrations of α-globulin in the thyroid tissue—here taken as an expression of the maximum amount of thyroglobulin which may occur in the tissue—are related to the amounts of thyroglobulin antibody in the serum The dispersion is not as great as for the thyroglobulin concentrations (Fig 1), and the values obtained are of the same order as the thyroglobulin concentrations which *DeGroot & Carvalho* (1960) revealed in experiments with ultracentrifugation However, Fig 3 does not either reveal any relationship between the α-globulin concentrations in the thyroid tissue and the thyroglobulin-antibody titre in the serum

In some of the cases, good agreement between the concentrations of thyroglobulin and α-globulin was observed However, this scarcely

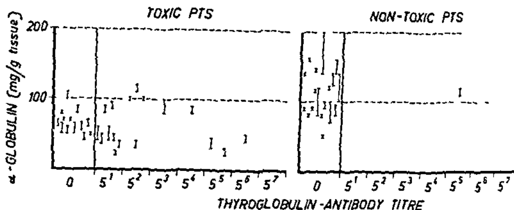


Fig 3
α-Globulin concentrations in thyroid tissue related to the amounts of thyroglobulin antibody in the serum

means that the α-globulin fractions and the thyroglobulin standard used have consisted of pure thyroglobulin, but it should rather be taken as indicating that the α-peak has here been of the same composition as the purified thyroglobulin fraction which was used as the standard (in the electrophoretic analysis, it migrated as an almost pure α-peak) However, in other cases, the two values showed considerable divergence In order to throw light on this discrepancy, the ratio of the thyroglobulin concentration (in cases in which this could only be fixed at an approximate level, a mean value was used) to the concentration of α-globulin was calculated $I = \frac{\text{concentration of thyroglobulin in thyroid tissue}}{\text{concentration of α-globulin in thyroid tissue}}$

These values will obviously be beset with a considerable uncertainty, which also appears from the fact that in some of the cases the two results which were obtained independently of each other from the same gland showed a fairly wide difference From Fig 4, in which the calculated ratio is related to the amount of thyroglobulin antibody in the serum, it is seen that considerable variations occur from patient to patient If, in vivo, the thyroglobulin antibody is bound to the thyroglobulin in the thyroid gland, it might be expected—even if the phenomenon may be equalized through an increased production of thyroglobulin—that the ratio was lower in patients with thyroglobulin antibody than in those without this antibody However, such a tendency is not seen in Fig 4 On the contrary, some of the highest values were found among the patients with thyroglobulin antibody, and only a few of these patients had a ratio of approx 1 CA-2-antibody was present in two of the three patients who seemed to form a separate group with exceptionally low ratios (below 0 6)

Thus, the results in Fig 4 might suggest that the thyroglobulin extracts from some of the patients, including most of those with thyroglobulin auto-immunization, have a relatively greater thyroglobulin-antibody-fixing capacity (in relation to the amount of α-globulin) than

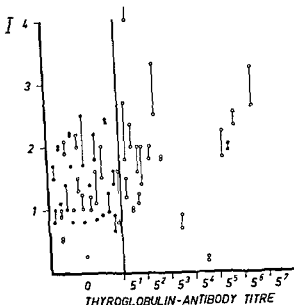


Fig 4

The ratio between the concentrations of thyroglobulin and α globulin in thyroid tissue (I) related to the amounts of thyroglobulin antibody in the serum
○ Toxic patients ● Non toxic patients

the extracts from the majority of the patients without thyroglobulin antibody This might be due either to the presence of antibody-fixing groups outside the α globulin fraction or to a changed composition of the α globulin fraction in the extracts, including a changed composition of the thyroglobulin itself

In an attempt to throw light on the last mentioned possibility a purified thyroglobulin fraction was prepared by the method of *Derrien et al* (1948) from each of 11 goitres originating from patients with or without thyroglobulin antibody After dialysis and freeze-drying, solutions containing from 2 to 20 μg of the thyroglobulin fractions per ml were prepared, and amounts of 0 1 ml of these solutions were mixed with 0 1 ml of diluted test serum (1 100) containing thyroglobulin antibody After standing at 37° C for 4 hours, thyroglobulin coated erythrocytes were added The resultant pattern of haemagglutination inhibition is shown in Table 7, in which the thyroglobulin antibody titre in the serum and the ratio (I) between the amounts of thyroglobulin and α globulin in the extracts studied are also listed These purified thyroglobulin fractions, which in electrophoretic analysis migrated as almost pure α peaks, did not show quite as great variations in their thyroglobulin antibody-fixing capacity as seen in Fig 4 However, in repeated determinations, the fractions from the four patients with the lowest antibody titres (Nos 7–10) differed unquestionably from the standard and from the fractions which originated from patients without thyro

globulin auto immunisation In No 8 (Table 7), which in spite of the presence of thyroglobulin antibody revealed almost equally large amounts of thyroglobulin and α-globulin in the two extracts studied, the thyroglobulin fraction thus fixed about 50 per cent more antibody than the standard fraction On the other hand, the two cases with higher antibody titres (Nos 11 and 12, Table 7) did not differ from the standard The experiment thus lends support to the view that variations may occur in the composition of the α-globulin fraction of the thyroid extracts, but the antibody-fixing capacity of the thyroglobulin fractions scarcely vary so much that it will be permissible to draw any conclusions as to a change in the composition of the thyroglobulin molecule itself

TABLE 7

The Antibody Fixing Effect of the Thyroglobulin Fractions from Various Glands

No	TGA titre	I	Amount of thyroglobulin fraction (µg o 1 ml) to which o 1 ml test serum was added (dil 1 100)									
			0 2	0 4	0 6	0 8	1 0	1 2	1 4	1 6	1 8	2 0
1	0	Standard	4 4	4 4	4 4	4 4	4 4	4 4	4 4	0 1	0 0	0 0
2	0*	0 25	4 4	4 4	4 4	4 4	4 4	4 4	4 4	4 4	4 4	3 3
3	0	0 8 1 0	4 4	4 4	4 4	4 1	4 4	4 4	2 3	0 0	0 0	0 0
4	0	0 8	4 4	4 4	4 4	4 4	4 4	4 4	4 4	0 0	0 0	0 0
5	0	1 1	4 4	4 4	4 4	4 4	4 4	4 4	2 0	0 0	0 0	0 0
6	0*	2 2 2 2	4 4	4 4	4 4	4 4	4 4	4 4	4 4	0 0	0 0	0 0
7	5	4 0–4 5	4 4	4 4	4 4	4 4	4 4	1 0	0 0	0 0	0 0	0 0
8	5	1 1 1 25	4 4	4 4	4 4	4 4	4 2	0 0	0 0	0 0	0 0	0 0
9	25	2 0–1 8	4 4	4 4	4 4	4 4	4 4	2 2	0 0	0 0	0 0	0 0
10	125	0 7 0 9	4 4	4 4	4 4	4 4	4 4	2 2	0 0	0 0	0 0	0 0
11	625	3 5 3 2	4 4	4 4	4 4	4 4	4 4	4 3	2 1	0 0	0 0	0 0
12	3125	1 8 2 2	4 4	4 4	4 4	4 4	4 4	4 4	3 2	0 0	0 0	0 0

The figures indicate the degrees of haemagglutination See Table 1
* *Serum contained CA 2 antibody*

DISCUSSION

The studies presented here did not suggest that the thyroglobulin antibody in vivo is capable of neutralizing the antigen in the thyroid gland This result does not agree with the observation made by *Rose &*

Witebsky (1956) after active immunization of rabbits by means of [illegible] extract to which *Freund*'s adjuvant was added In their [illegible] immu- [illegible] of circulating antibody and the decrease in the antigen [illegible] served The discrepancy may perhaps be explained by the active immunization of the rabbits by the use of *Freund*'s adjuvant (with development of delayed hypersensitivity?)

That it was necessary here to employ the rather troublesome roundabout method of comparing the amount of free, thyroglobulin-antibody-*fixing groups in the thyroid* tissue with that of α globulins, is due to the fact that it was not possible to produce an absolutely pure thyroglobulin (*Shulman & Witebsky* 1960), and just this fact renders the evaluation of the results difficult As regards the relation between the amounts of thyroglobulin-antibody-fixing groups and α globulin, the composition of the extracts seems to vary within fairly wide limits The results obtained are compatible with the view that the relative content of thyroglobulin groups seems to be able to increase from a certain basic level, which is found mainly in patients without thyroglobulin auto-immunization, to a higher level which occurs in some patients without signs of production of *antibody against thyroglobulin, but especially in patients* in whom thyroglobulin antibody is present On the other hand, a level which was appreciably lower than the basic one was observed in three patients, including two who had CA 2 antibody in the serum (The relatively low thyroglobulin content might perhaps be due to a relatively higher content of second colloid antigen)

It is a well known fact that the complement-fixing antigen is present in the greatest amounts in glandular tissue from thyrotoxic patients (*Roitt &Doniach* 1960), and apparently also occurs in a high concentration in the active glandular tissue in Hashimito's disease (*Pulvertaft et al 1961*) Thus, the greatest amounts of antigen are seen in the groups of patients in whom the occurrence of the corresponding complement fixing antibody is most frequent Accordingly, it would seem reasonable to assume that *patients with production of antibody against* thyroglobulin must be sought among those with a relatively high thyroglobulin antibody-fixing (antigenic?) activity in the glandular tissue A higher antigen level might be either the result of the immunization processes (an overcompensation phenomenon?) or the cause of the development of auto-immunization The fact that the complement fixing antigen may be present in a high concentration in the tissue without being accompanied by complement fixing antibody in the serum, and that a high thyroglobulin activity may occur in the absence of thyroglobulin antibody, weighs against the former possibility.

Both as *far as the complement-fixing* antigen and thyroglobulin are concerned, it applies that the higher antigen level might be the result either of purely quantitative changes in the composition of the proteins

of the thyroid gland or of qualitative changes, so that the individual antigen molecule might contain more antibody-fixing (antigenic?) groups than usual. The latter possibility would explain the development of auto-immunization, i.e. the disease would be the primary and the auto-immune reaction a secondary phenomenon caused by changes in the antigen structure. These considerations are based on studies on the conditions in patients with thyrotoxicosis or non-toxic goitre, and the assumptions do therefore not exclude the view that the auto-immune reactions in other thyroid disorders—lymphadenoid goitre, primary myxoedema—might be of a primary pathogenic significance and be directed against normal antigen molecules. If absolutely pure antigen could be produced, it would be easy to decide if both possibilities might actually occur.

SUMMARY

In 71 patients who underwent operations on the thyroid gland, attempts were made to obtain relative, but comparable measures for the thyroglobulin concentrations in the thyroid tissue by means of an immunological method, viz. a haemagglutination-inhibition technique, which is described. At the same time, the patients were studied for thyroglobulin antibody, complement-fixing thyroid antibody and CA 2-antibody. No correlation between the thyroglobulin concentrations in the thyroid tissue and the occurrence of thyroid auto-antibodies in the serum could be demonstrated.

As thyroglobulin in electrophoresis migrates as α-globulin, the content of α-globulins in the thyroid tissue was determined in a number of the cases in order to obtain a measure of the maximum amount of thyroglobulin which might occur in the tissue. In some cases, good agreement was found between the amounts of thyroglobulin determined by the immunological method and the content of α-globulin, while other cases—especially patients in whom thyroglobulin antibody was demonstrated in the serum—showed a discrepancy between these two values, the thyroglobulin concentrations relatively being too high.

As the purified thyroglobulin fractions also showed some variations in their thyroglobulin antibody fixing capacity, it is discussed if the exciting factor in thyroglobulin auto-immunization may be a change in the composition of the thyroglobulin depot of the thyroid gland, or if a change in the thyroglobulin molecule may occur so that it contains more antigenic groups than usual.

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FROM THE INSTITUTE OF HISTOLOGY AND THE INSTITUTE OF PATHOLOGY
UNIVERSITY OF GOTEBORG SWEDEN

TISSUE REACTION TO BARIUM SULPHATE CONTRAST MEDIUM

By

HANS ROCKERT and LENNART ZETTERGREN

Received [illegible] 1 63

Over a number of years we have now and then noticed peculiar granulomatous lesions in the abdomen Correlation of clinical and pathological data in such cases suggested that the granulomatous changes were caused by barium sulphate which after X ray investigation had leaked through a peptic ulcer either in the gastric or duodenal wall into the peritoneal cavity In all the cases studied the granulomatous changes were chiefly located in the greater omentum As the lesion differs from barium granulomas previously reported in the literature we shall describe the principal features of one of our cases where the phagocyting cells in the granulomas have been investigated and analyzed with the aid of light microscopy contact microradiography and X ray microfluorescence

The tissue block investigated was obtained from a man 58 year old who had suffered from gastric pains for about 10 years After [illegible] ack [illegible] ion [illegible] we [illegible] the peritoneal cavity By operation ad modum Billroth + enteroanastomosis two thirds of the gastric wall and part of the duodenum was removed

LIGHT MICROSCOPY

Microscopically a peptic ulcer was evident in the duodenal wall In the connective tissue around the duodenum and in the omental fat could be seen areas with large cells well filled with cytoplasm containing small nuclei and dense chromatin (Fig 1) On staining with haematoxylin—v Gieson the cytoplasm of the cells was greyish and showed fine granules which clearly refracted the [illegible]

[illegible] lymphocytes or plasma cells No barium crystals were seen microscopically, nor had contrast medium been [illegible]

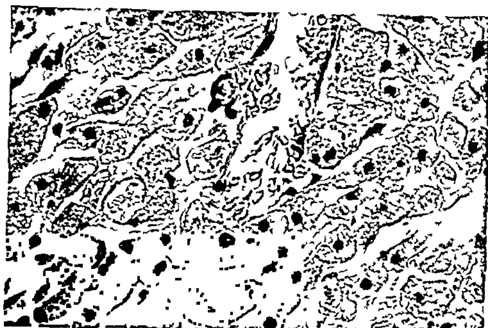


Fig 1

Cell containing cytoplasmatic barium granules seen in transmitted light From the subserosal connective tissue in the gastric wall

sible to the naked eye *P A D Ulcus pepticum chron perforatum* (?) *duodeni, bariumgranulomas*

CONTACT MICRORADIOGRAPHY

I Sections 7 μ in thickness from the paraffin embedded tissue were placed in close contact with a film emulsion (Kodak Spectroscopic Plate No 649) They were then exposed to X rays for three minutes at 10 KV and 10 mA (Machlett X ray tube OLG 50A, Cu anode, 1 5 mm focal spot 1 mm beryllium filter) The cells described in the previous section appeared on the film to have white cytoplasm, due to the high X ray absorption of this part of the cell, in contrast to the general tissue background The nucleus did not absorb any X ray and appeared as a black spot in the cytoplasm when exposed to X rays within the wave length region used, 1 2 4 Å (Fig 2)

II Similar sections, with a thickness of 5 μ, cut from the same tissue block were exposed to X rays within the wave length region 8–10 Å using the same technique (Cu anode 3 5 KV 3 5 mA, exposure time 15 minutes) The micro radiograms obtained show the mass distribution within the section, according to *Engstrom & Lindstrom* (1950) The microradiograms could also be used for location of the highly X ray absorbing cells in the organic stroma within the section (Fig 3)

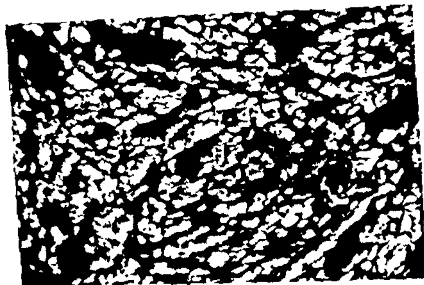


Fig 2

Microradiogram obtained in the soft X ray region (see text) from cells containing barium sulphate From the same area as in Fig 1



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X RAY FLUORESCENCE MICRO ANALYSES

About five isolated cells (dissected out by hand under a binocular microscope) were bombarded with X-rays from an X-ray microscope (type Cosslett Nixon, Cu anode, 15 kV, 150 uA) The cells were placed

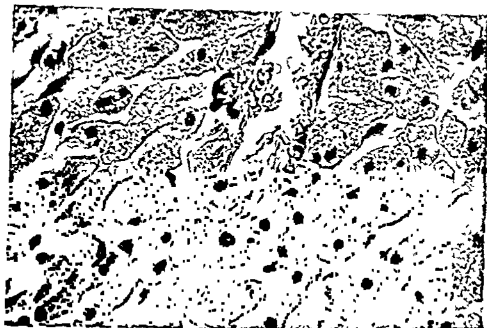


Fig. 1.
Cell containing cytoplasmatic barium granules seen in transmitted light
From the subserosal connective tissue in the gastric wall

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II Similar sections, with a thickness of 5 μ, cut from the same tissue block were exposed to X-rays within the wave length region 8–10 Å, using the same technique (Cu anode, 3 5 KV, 3 5 mA, exposure time 15 minutes) The micro-radiograms obtained show the mass distribution within the section, according to *Engstrom & Lindstrom* (1950) The microradiograms could also be used for location of the highly X-ray absorbing cells in the organic stroma within the section (Fig 3)

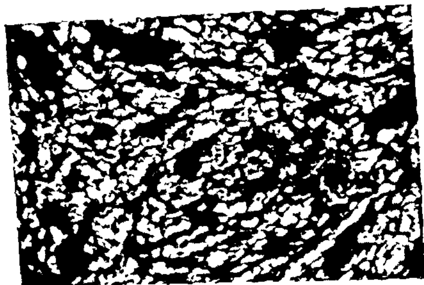


Fig 2

Microradiogram obtained in the soft X ray region (see text) from cells containing barium sulphate From the same area as in Fig 1



Fig 3

Microradiogram obtained in the ultrasoft X ray region showing the mass distribution within the section The area with *barium containing* cells is seen to the right

X RAY FLUORESCENCE MICRO ANALYSES

About five isolated cells (dissected out by hand under a binocular microscope) were bombarded with X rays from an X ray microscope (type Cosslett Nixon, Cu anode, 10 kV, 100 µA) The cells were placed



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berger 1928, *Bittrolff* 1928, *Himmelmann* 1932, *Singer* 1934, *Strasser* 1946, *Kay* 1954) Most of these cases have had peptic ulcers in the gastric or duodenal wall which have perforated either before or during the X ray investigation In a minor number of cases leakage of contrast medium has occurred through the appendix, colic, sigmoid or rectal walls during the administration of barium enemata

The tissue reactions caused by the leakage of barium sulphate from the digestive tract have been studied by a few authors (*Kay* 1954 and *Mendeloff* 1956) The effect of endotracheal and intraperitoneal injection of barium sulphate suspensions has been studied under experimental conditions by *Kleinsasser & Warshaw* (1952) and Huston, Wallack & *Cunningham* (1952)

The barium granulomas seen in our material differ somewhat from those describ
particles desc
by the latter
(Mixobar) is composed of very fine particles and that the small grains are probably all caught by cell phagocytosis

In our experience barium granulomas may sometimes cause the pathologist diagnostic difficulty In one of our cases the greyish white areas were considered by the surgeon as talc granulomas following a previous operation on the abdomen Sometimes the clinical data available to the pathologist are not of the kind which might give him an association leading to the correct diagnosis

In order to get an idea of how long the barium granulomas might be detectable after their development white rats were given 0 8 cm² barium sulphate (Mixobar) intraperitoneally A week after the injection granulomatous spots were seen in the fat tissue of the greater omentum and in the peritoneum Microscopically the spots were all like barium granulomas in man One rat was allowed to live seven months after the injection and small greyish white plaques were seen in the peritoneal cavity The refringence and the granules of the cells in this instance were less distinct than those from fresh granulomas and the microscopic picture showed fewer granules in the cytoplasm

SUMMARY

Details of a case of barium granuloma in the peritoneal cavity, caused by leakage of barium contrast medium (Mixobar) through a peptic ulcer in the duodenum are described The granuloma showed mainly large cells containing cytoplasm filled with small refringent granules The cells were investigated by contact microradiography, and X ray fluorescence micro-analysis and proved to contain barium sulphate Comparison of the X ray spectrum of the intracellular granules and a sample of barium contrast medium used for clinical diagnostic purposes proved to be identical

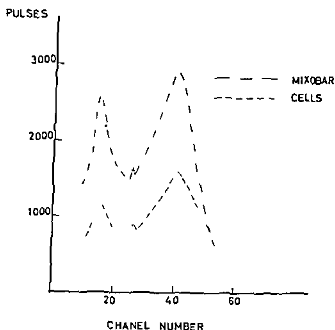


Fig 4
X ray fluorescence curves as seen on the oscilloscope Top curve from the contrast medium, bottom curve from the cells

on top of an electron microscope aperture with a diameter of 200 μ in an evacuated chamber Within the specimen secondary X rays were generated, *i e* X-ray fluorescence radiation, whose spectral definition is due to the atomic composition of the specimen The intensities at different wavelength of the fluorescence radiation were analyzed with the aid of a proportional counter and a pulse height multichannel analyzer and made visible on an oscilloscope A curve was obtained and along the x-axis different energies of wavelengths were plotted Along the ordinate the height of the peaks of the curve represented the intensity at a particular wavelengths *i e* the amount of an element Each element has been shown to have a separate peak (*Long & Rockert* 1963)

Using this method the cells analyzed produced peaks at wavelengths corresponding to the emission peaks of barium (Fig 4) A comparative investigation made with a small drop of Mixobar, the same contrast medium used for the clinical gastric X-ray examination, produced peaks identical with those from the cells The only difference was the height of the peaks due of course to the larger amount of material analyzed This confirmed that the cytoplasm of the cells analyzed contained barium contrast medium

DISCUSSION

Leakage of barium contrast medium into the abdominal cavity during gastric X-ray examination has been shown in number of cases (*Am-*

berger 1928, *Billroff* 1948, *Himmelmann* 1932, *Singer* 1934, *Strasser* 1946, *Kay* 1954) Most of these cases have had peptic ulcers in the gastric or duodenal wall which have perforated either before or during the X ray investigation In a minor number of cases leakage of contrast medium has occurred through the appendix, colic, sigmoid or rectal walls during the administration of barium enemata

The tissue reactions caused by the leakage of barium sulphate from the digestive tract have been studied by a few authors (*Kay* 1954 and *Mendeloff* 1956) The effect of endotracheal and intraperitoneal injection of barium sulphate suspensions has been studied under experimental conditions by *Kleinsasser* & *Warshaw* (1952) and *Huston, Wallack* & *Cunningham* (1952)

The barium granulomas seen in our material differ somewhat from those described in the literature We have not seen the extracellular particles described by *Kay* and *Mendeloff*, nor the giant cells described by the latter The reason for this may be that the barium sulphate used (Mixobar) is composed of very fine particles and that the small grains are probably all caught by cell phagocytosis

In our experience barium granulomas may sometimes cause the pathologist diagnostic difficulty In one of our cases the greyish white areas were considered by the surgeon as talc granulomas following a previous operation on the abdomen Sometimes the clinical data available to the pathologist are not of the kind which might give him an association leading to the correct diagnosis

In order to get an idea of how long the barium granulomas might be [illegible] given 0 8 cm² ba- [illegible] after the injection [illegible] greater omentum and in the peritoneum Microscopically the spots were all like barium granulomas in man One rat was allowed to live seven months after the injection and small greyish white plaques were seen in the peritoneal cavity The refringence and the granules of the cells in this instance were less distinct than those from fresh granulomas and the microscopic picture showed fewer granules in the cytoplasm

SUMMARY

Details of a case of barium granuloma in the peritoneal cavity, caused by leakage of barium contrast medium (Mixobar) through a peptic ulcer in the duodenum, are described [illegible] ainly large [illegible] nules The [illegible] ay fluores- [illegible] Compari[illegible] [illegible] spectrum of the intracellular granules and a sample of barium contrast medium used for clinical diagnostic purposes proved to be identical

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FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI, HELSINKI, FINLAND

ADENOSINE TRIPHOSPHATASE ACTIVITY OF RAT SKIN IN EARLY WOUND HEALING

By

J. RAEKALLIO and EEVA LEVONEN

Received 24.1.63

In previous investigations the early appearance of some hydrolytic and oxidative enzymes (*Raekallio* 1960, 1961) and of transglucosylases (*Raekallio & Levonen* 1962) has been demonstrated in healing wounds. Among the specific phosphatases adenosine triphosphatase (ATPase) plays an essential rôle in various metabolic processes. In this investigation, therefore, its appearance and distribution in the skin has been studied during the first 24 hours of wound healing.

MATERIAL AND METHODS

[illegible] lized with [illegible] ounds [illegible] inter-vals of 1, 2, 4, 8, 16 and 24 hours after wounding. Skin flaps containing the wounds were removed immediately and frozen with solid carbon dioxide for sectioning in a cryostat.

Sections were cut at 15 μ, fixed for 10 minutes in ice cold neutral formol calcium (Novik [illegible] et al. 1961), washed in one per cent calcium chloride solution for two minutes, incubated for 15 minutes in a substrate solution of *Padykula & Herman* (1955) and further processed according to their method. For control sections a glycerophosphate was used instead of ATP in the substrate solution.

RESULTS

Brown black deposits of lead sulphide indicated ATPase activity in the sections. In the uninjured skin farther away from the wound moderate to intense ATPase activity was observed in occasional fibroblasts. Most of them were situated in the papillary layer of the dermis, especially in the vicinity of the dermoepidermal junction where the capillaries also showed intense activity. The panniculus carnosus and the arrectores pilorum muscles were intensely or moderately stained. Ve-

This work was facilitated by grants from the Emil Aaltonen Foundation to the authors, and from the Sigrid Jusélius Foundation to the department.

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In previous investigations the early appearance of some hydrolytic and oxidative enzymes (*Raekallio* 1960, 1961) and of transglucosylases (*Raekallio & Levonen* 1962) has been demonstrated in healing wounds Among the specific phosphatases adenosine triphosphatase (ATPase) plays an essential role in various metabolic processes In this investigation therefore, its appearance and distribution in the skin has been studied during the first 24 hours of wound healing

MATERIAL AND METHODS

Four month old white rats weighing approx 200 g

Sections were cut at 15 µ fixed for
(*Novikoff et al* 1961) washed in on
minutes incubated for 15 minutes in
(1955) and further processed accor
α glycer phosphate was used instead

RESULTS

Brown black deposits of lead sulphide indicated ATPase activity in the sections In the uninjured sk
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Fig 1

ATPase activity in an 1 hour-wound On the right the central zone exhibits a diminishing activity except in the hair follicle In the peripheral wound zone, the fibroblasts increasingly active in the upper dermis × 150

tive hair follicles exhibited an intense reaction for ATPase in the upper bulb The root sheaths were moderately stained So also were the basal and granular layers of the epidermis The stratum spinosum showed less conspicuous activity and no reaction at all was observed in the horny layer In the peripheral cells of the sebaceous glands moderate activity could be seen, whereas it decreased progressively in the cells undergoing sebaceous transformation

In the immediate vicinity of the injury, the ATPase activity of the dermal fibroblasts and of the capillaries had disappeared in the 200 μ to 500 μ deep central wound zone after only one hour (Fig 1) The activity of the hair follicles, however, was maintained during the experimental period

Peripherally to the central zone the first signs of increase in the activity of the fibroblasts were noted after one hour (Fig 1) The reaction became more marked in 2- (Fig 2) and 4 hour wounds After eight hours (Figs 3 and 4) the activated fibroblasts and invading, chiefly polymorphonuclear, leucocytes formed a distinct zone The leukocytes were more numerous in the vicinity of the panniculus carnosus In 16- and 24-hour wounds the peripheral zone, 100 to 300 μ in depth, consisted of migrant cells, chiefly mononuclears and of intensely active fibroblasts and endothelial cells

In the panniculus carnosus ATPase activity diminished gradually



Fig 2

A 2 hour wound ATPase active fibroblasts are seen in the peripheral zone including the lower dermis × 150

toward the wound edge where the area of necrotizing muscle bundles was occupied by monouclear cells after 16 to 24 hours

In the control sections incubated with α glycerophosphate instead of ATP as substrate strong non specific alkaline phosphatase activity somewhat resembled that of ATPase, but the first signs of increased activity did not appear until after four hours in the peripheral wound zone

DISCUSSION

To the best of our knowledge there has been no previous report concerning the overall distribution of ATPase activity in the intact skin of the rat or any other animal According to our results, a reaction for ATPase could be demonstrated at the sites of intense metabolic processes, as in the proximal layers of the epidermis, in the upper bulb



Fig 3
An 8-hour-wound where the invading leucocytes in the lower part contribute to the ATPase activity of the peripheral zone × 150

and the root sheaths of the active hair follicles, in some fibroblasts, and, of course, in the muscle tissue

In conformity with other enzyme reactions in wounds (*Raekallio* 1961, 1963), the decrease in ATPase activity in the control zone, nearest to the wound edge, should be considered as an early sign of imminent necrosis The hair follicles seem to be more resistant than the dermal elements

An increase in ATPase activity was observed in the peripheral wound zone as early as one hour after the injury This activity then continued to intensify It is notable that the fibroblasts were the first to be activated *Montagna* (1962) has suggested that the fibroblast is the stem cell from which all other cells of the connective tissue arise, including those with a phagocytic function According to *Senda* (1962) phagocytosis is performed by a mechano-chemical system which is connected with ATPase Thus, the early activation of ATPase in fibroblasts may reflect, *inter alia*, a shift of their function towards phagocytosis On the other hand, according to *Braunstein et al* (1962), enzymatic reactions are generally more marked in proliferating cells In addition, *Lennert & Rinneberg* (1961) have observed ATPase activity at the sites of vigorous fibre formation Accordingly, the intensified activity of this



Fig 4

Part of Fig 3 showing the peripheral wound zone with active fibroblasts and leucocytes In the upper right hand corner of the figure strongly stained capillaries are seen The ATPase inactive central zone is in the lower right hand corner ×450

enzyme in the periphery of wounds may be an indication of this proper function of fibroblasts in a later phase of healing Invasion by leucocytes was clearly demonstrable 8 hours or more after wounding According to Senda (1962) both the mobility and the phagocytic function of leucocytes are related to ATPase dependent biochemical reactions This may explain the intense ATPase activity of the invading cells

In a later phase of healing the non specific alkaline phosphatase and ATPase activities exhibit an almost similar pattern in the wound periphery At this stage it is difficult to distinguish between these two activities In the hours immediately subsequent to wounding however the activation of ATPase in the fibroblasts represents the earliest histochemically proved response to injury

SUMMARY

Adenosine triphosphatase (ATPase) activity of uninjured and wounded rat skin was studied by using the histochemical method of *Padykula & Herman*. In the uninjured skin specific ATPase activity was observed in the proximal layers of the epidermis, in the dermal fibroblasts, in the arrectores pilorum muscles and in the panniculus carnosus. It is apparently present, together with non-specific alkaline phosphatase, in the active hair follicles and in the periphery of the sebaceous glands. In the fibroblasts of the wound periphery a distinct activation of ATPase was noted after only one hour, by which time the enzyme had disappeared from the innermost zone. After eight hours a pronounced peripheral wound zone had been formed containing ATPase-active fibroblast and invading leucocytes.

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EXPERIMENTAL TOXOPLASMOSIS

Parasitemia in guinea-pigs

By

GUNNEL HULDT

Received 2 x 62

In a previous paper (4) it was shown that experimentally infected guinea pigs can transmit toxoplasmosis to their offspring relatively late after inoculation It was also demonstrated that in two cases Toxoplasma could be isolated from the blood 13 and 4 weeks, respectively, after inoculation despite the presence of antibodies in the blood It seems most likely that the parasitaemia in these cases was transient

Toxoplasma gondii has been demonstrated in the blood of man and different animals by a number of investigators Studies of parasitaemia in naturally infected pigeons were made in 1949 by *Feldman & Sabin* (2) and in 1952 by *Jacobs* and coworkers (6) In studies of experimental infection in pigeons *Jacobs* and coworkers (7) showed that pigeons contract a heavy parasitaemia which starts 3–8 days after infection and continues until the animal dies, usually 3 weeks after inoculation

A systematic study of parasitaemia in various mammals was carried out by *Jacobs & Jones* (5) in 1950 The authors showed that in experimentally infected mice and rabbits parasites could be isolated from the blood 3 4 days after inoculation, and that there was a progressive increase in parasitaemia level until the death of the animal from toxoplasmosis Mice with asymptomatic toxoplasmic infection exhibited lower blood parasite levels in the early period following inoculation, after which parasites were found in the blood only rarely and in small numbers A low blood parasite level was also found in one rabbit which survived infection Experimentally infected rats presented only mild and transient symptoms and showed very low and sporadic parasitaemia

In studies published in 1961 *Remington, Melton & Jacobs* (12) showed that parasitemia can occur spontaneously in mice, rabbits and guinea pigs chronically infected with certain strains of Toxoplasma [illegible]

[illegible] parasitemia

In man parasitaemia has been demonstrated in a number of cases (1 9 10, 11, 13, 15) of both congenital and acquired toxoplasmosis

Toxoplasma organisms have been found not only in severe but also in asymptomatic cases of acquired toxoplasmosis (11). In our laboratory Toxoplasma was isolated *from the blood* in three human cases of acquired toxoplasmosis all of moderate severity (main findings, lymphadenopathy in two cases, myocarditis in one case). In all three cases Toxoplasma was isolated during the first month of the illness, however, antibodies in high titre were found in the same blood sample.

In the present work the parasitaemia of infected guinea-pigs has been studied with respect to the time of its appearance, its duration, and the influence of different infective doses as well as of different modes of infection. Finally, previously infected guinea-pigs have been reinoculated in order to study whether the first infection induced immunity sufficient to protect the animal against new parasitaemia.

MATERIAL

Experimental animals. Seventy eight guinea pigs were used for the experiments. All animals were young, around 6 months of age (weight 400–500 gm), and of the same breed. They were keept strictly segregated in individual cages and were fed during the experiment only on pellets and Swedish turnips. All lacked antibodies before inoculation according to dye and complement fixation test.

Mice of a well controlled breed free of toxoplasmosis were used for isolation experiments and for providing antigen for the dye test. The mice used for isolation experiments weighed 12–14 gm and for the dye test 16–18 gm.

Toxoplasma strain. Sabin's strain RH was used throughout.

METHODS

Inoculation of guinea pigs. Fresh peritoneal exudate from mice with toxoplasmosis was used for inoculating the guinea pigs. The Toxoplasma organisms in the exudate were first counted in a Buerker chamber, upon which the exudate was *diluted to* contain the desired amount of parasites. The inoculum was administered subcutane
... or intranasally (0.2 ml).
... investigation the dye test (*DT*) and *the*
... The methods employed in this laboratory
... is paper (4).

The blood was drawn by cardiac puncture. If blood was needed both for serological *investigation and for isolation experiments 5 ml were drawn, for isolation experi*ment 2.5 ml were drawn.

Isolation experiments. The Sim Aagaard technique (13) was used. The isolations were carried *out with heparinized* whole blood, undiluted and in the dilutions 1/10, 1/100 and 1/10000. 0.5 ml of the material was injected intraperitoneally into mice (5 mice were used for each dilution) immediately after cardiac puncture of the guinea pig.

EXPERIMENTAL PROCEDURE AND RESULTS

Parasitaemia in experimentally infected guinea-pigs was studied in five separate experiments. *Three main problems* were investigated, namely the appearance and early development of parasitaemia in *DT* and CFT-negative animals (group A, B), the duration of parasitaemia in such animals (group *C*, *D*), and finally the *protective effect against* parasitaemia obtained by immunization with living parasites (group E).

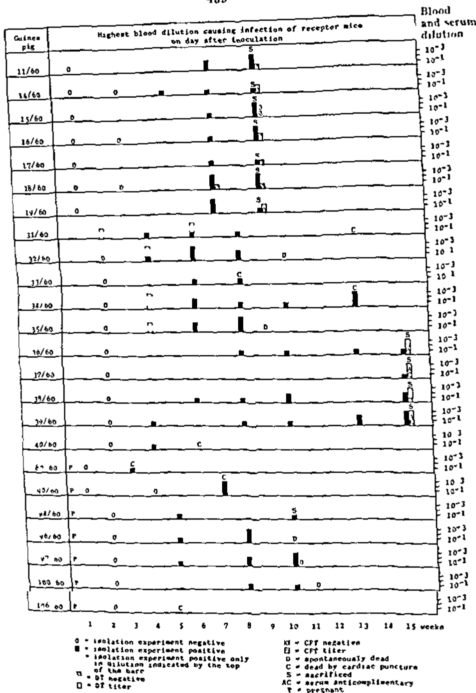


Fig 1

Group A 22 guinea pigs inoculated subcutaneously with 5 000–10 000 toxoplasmas Blood and serum dilutions on log scale

Group A

Parasitaemia during the first two weeks after inoculation was followed For this purpose 24 guinea pigs, 7 of which were pregnant, were inoculated with 5,000–10,000 toxoplasma organisms Isolation experiments on heart blood and dilutions were made every two days (on a few occasions every 3 days) on the non-pregnant animals, beginning with the first day in 9 animals and with the second day in 8 animals In the pregnant guinea-pigs isolation experiments were carried out less frequently (every 3 days)) because of the sensitivity of these animals to cardiac puncture During the experiment 12 animals died The remaining 12 animals were sacrified, 7 on the ninth day after inoculation, one on the tenth day, and 4 on the fifteenth day Immediately before the animals were killed, blood for isolations, DT, and CFT was taken

Fig 1 shows the appearance of Toxoplasma in the blood and the blood parasite level in the individual animals The DT and CFT in blood taken immediately before the animals were sacrificed is also demonstrated One of 15 animals investigated had parasitaemia on the second day after inoculation, 5 of 7 on the third day, and 10 of 11 on the fourth day On the fifth day Toxoplasma was isolated from all of the blood samples in dilutions 1/1–1/1000 In 5 isolation experiments Toxoplasma was detected in one high blood dilution whereas one or two lower dilutions were negative This phenomenon will be discussed later *The blood*

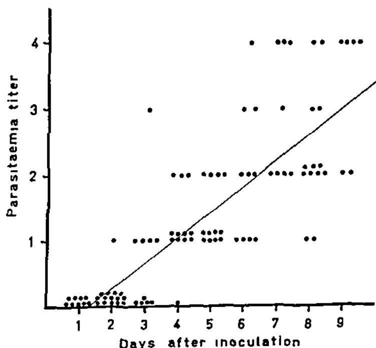


Fig 2

Guinea pigs group A Blood parasite level on days after inoculation *Ordinate* Dilutions of blood from which toxoplasma were isolated Blood dilutions on log scale Significance of symbols are indicated in the text

parasite level in these cases is considered to be represented by the highest positive dilution with all the lower dilutions positive

In Fig 2 the individual isolations are recorded The diagram covers only 9 days of the experiment, because after the ninth day only 9 of 22 animals were alive, the material was therefore no longer sufficiently representative It can be seen from the diagram that an increase in blood parasite level set in on the third day after inoculation and proceeded during the time recorded This increase is highly significant, *the variation quotient*, $V^2 = 165$, $p < 0.001$

From Fig 1, it can be seen that parasitaemia did occur despite the presence of antibodies in high titre in the blood This was better demonstrated in groups C and D

In group A no marked difference in parasitaemia level was seen between animals with clinical and subclinical toxoplasmosis in contrast to Jacob s findings in mice and rabbits Of the three non pregnant animals that died spontaneously two (32.60, 38 60) had parasitaemia in high titre while in the third (10 60) parasitaemia could be demonstrated only in low titre On the other hand high titres were demonstrated in animal 34 60 which showed no signs of disease during the experiment However the numbers are insufficient to permit further conclusions in this respect

Parasitaemia in pregnant guinea pigs followed the same pattern as in non pregnant animals

Group B

In order to investigate whether a small infective dose might yield a lower degree of parasitaemia, a group of 9 guinea pigs were injected with a much smaller dose than that used in group A 100 200 parasites were injected subcutaneously in each of 9 guinea pigs Isolation experiments on heart blood were made every second day beginning with the first day after inoculation Eight animals died within the interval from day 7 to day 13 of the experiment One animal was sacrificed on the fourteenth day after inoculation Serological investigation after inoculation was not carried out in this group

From Fig 3 can be seen that in all cases Toxoplasma was isolated [illegible] after inoculation, at which [illegible] he seventh day Toxoplasma [illegible] ls The blood parasite level reached the same values in this group as in experimental group A, in which the animals had been given a heavier infective dose

In Fig 4 the individual titre values on different days after inoculation are recorded It can easily be seen that a marked increase in blood parasite level began on the fifth day after inoculation and continued during the time covered by the diagram i e, 11 days Highly significant values were also found in this material $V^2 = 175$ $P < 0.001$ There is

Group A

Parasitaemia during the first two weeks after inoculation was followed For this purpose 24 guinea pigs, 7 of which were pregnant, were inoculated with 5,000–10,000 toxoplasma organisms Isolation experiments on heart blood and dilutions were made every two days (on a few occasions every 3 days) on the non-pregnant animals, beginning with the first day in 9 animals and with the second day in 8 animals In the pregnant guinea-pigs isolation experiments were carried out less frequently (every 3 days)) because of the sensitivity of these animals to cardiac puncture During the experiment 12 animals died The remaining 12 animals were sacrificed, 7 on the ninth day after inoculation, one on the tenth day, and 4 on the fifteenth day Immediately before the animals were killed, blood for isolations, DT, and CFT was taken

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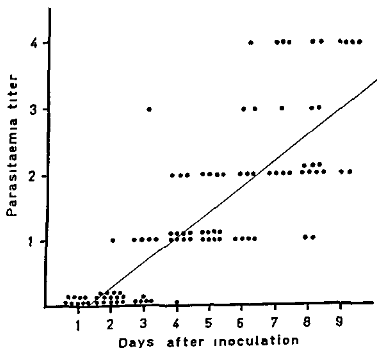


Fig 2

Guinea pigs group A Blood parasite level on days after inoculation Ordinate Dilutions of blood from which toxoplasma were isolated Blood dilutions on log scale Significance of symbols are indicated in the text

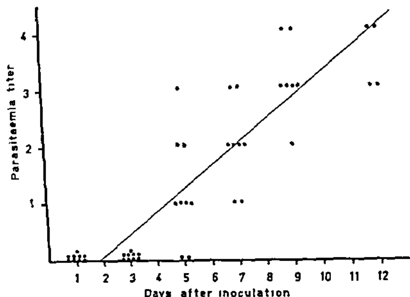


Fig 4

Guinea pigs group B Blood parasite level on days after inoculation Ordinate: Dilutions of blood from which toxoplasma were isolated Blood dilutions on log scale Significance of symbols are indicated in the text

Toxoplasma was isolated from the blood of only one of 12 animals investigated As from the fourth week transient parasitaemia was occasionally demonstrated in two animals In one case, in which isolations from the blood had been negative for seven weeks Toxoplasma was isolated from the blood 15 weeks after infection (Fig 5)

Compared with groups A and B the blood-parasite titres in group C showed lower values throughout In most cases the blood-parasite level was highest in samples taken one week after inoculation The transient parasitaemia occasionally appearing after the third week did not reach higher titres than 1 1 except in 2 cases 4 59, in which the titres was 1 100 7 weeks after inoculation, and 24 59 in which the titre was 1/100 5 weeks after inoculation

From Fig 5 it can be seen that in several cases Toxoplasma could be isolated from blood containing antibodies in high titre No marked difference in blood-parasite level between clinical and subclinical cases of toxoplasmosis can be seen in this material either

Group D

The experiments were performed one year erlier than those of the preceding group The main purpose was to study the duration of parasitaemia after intracutaneous and intranasal inoculation

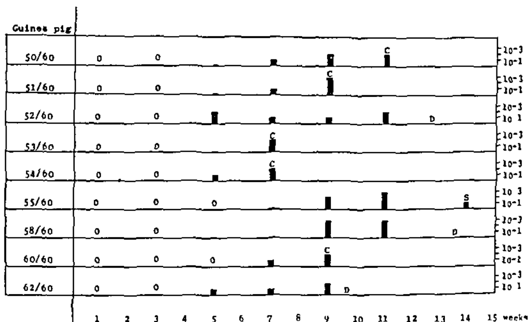


Fig 3

Group B 9 guinea pigs inoculated subcutaneously with 100–200 toxoplasmas Blood dilutions on log scale

no important difference between the diagrams in Figs 2 and 4 except the later appearance of parasitaemia in Fig 4 where the animals had obtained a smaller infective dose

A comparison between the blood parasite level in cases with *clinical* and subclinical toxoplasmosis does not show any obvious differences Guinea-pig 58 60 which died spontaneously had high parasitaemia titres in the last two blood samples However, titres of the same value were found in animal 55/60 which did not show any signs of disease Moreover, in the animals 52/60 and 62/60 which also died spontaneously Toxoplasma was isolated from the blood only in low and moderate titres

Group C

The aim of this experiment was to study the duration of parasitaemia Seventeen guinea-pigs were injected subcutaneously with 5,000–10 000 parasites Isolation experiments were carried out on heart blood and dilutions thereof once every week after inoculation, starting 7 days after inoculation in 13 animals and 14 days in 4 animals DT, CFT, and isolations were carried out on the same blood sample During the first 7 weeks 15 animals died from heart puncture or disease The two surviving animals were sacrificed 7 and 15 weeks after inoculation

One week after inoculation all investigated animals had parasitaemia After two weeks 9 of 17 animals had parasitaemia After three weeks

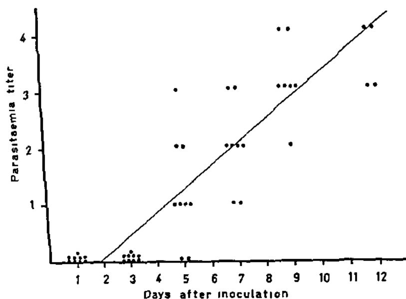


Fig 4

Guinea pigs group B Blood parasite level on days after inoculation Ordinate Dilutions of blood from which toxoplasma were isolated Blood dilutions on log scale Significance of symbols are indicated in the text

Toxoplasma was isolated from the blood of only one of 12 animals investigated As from the fourth week transient parasitaemia was occasionally demonstrated in two animals In one case, in which isolations from the blood had been negative for seven weeks Toxoplasma was isolated from the blood 15 weeks after infection (Fig 5)

Compared with groups A and B the blood-parasite titres in group C showed lower values throughout In most cases the blood parasite level was highest in samples taken one week after inoculation The transient parasitaemia occasionally appearing after the third week did not reach higher titres than 1/1 except in 2 cases 4/59, in which the titres was 1 100 7 weeks after inoculation, and 24 59 in which the titre was 1/100 5 weeks after inoculation

From Fig 5 it can be seen that in several cases Toxoplasma could be isolated from blood containing antibodies in high titre No marked difference in blood parasite level between clinical and subclinical cases of toxoplasmosis can be seen in this material either

Group D

The experiments were performed one year erlier than those of the preceding group The main purpose was to study the duration of parasitaemia after intracutaneous and intranasal inoculation

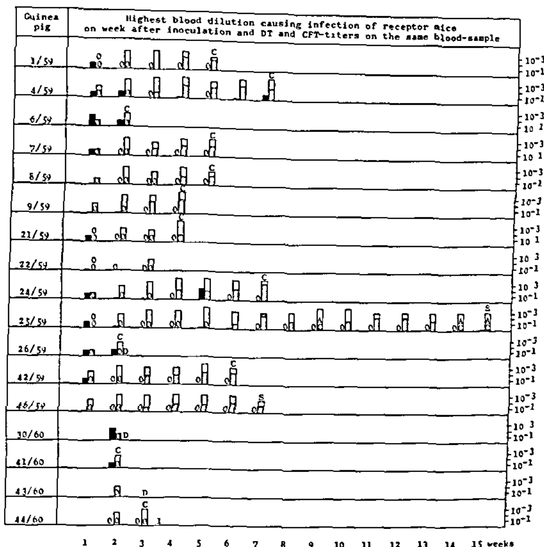


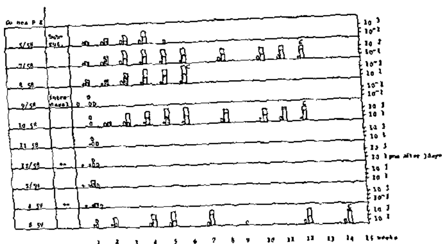
Fig 5

Group C 17 guinea pigs inoculated subcutaneously with 5 000 10 000 toxoplasmas
Blood and serum dilutions on log scale

Three animals were injected intracutaneously with *0 1 ml* of a suspension containing 400,000–800,000 parasites per ml (infective dose 40,000 80,000 toxoplasmas)

In seven animals 0 1 ml of a suspension *containing 200,000–400,000* toxoplasmas per ml was administered into each nostril Thus the infective dose given to the intracutaneously infected animals and to those infected intranasally was the same

Isolation experiments were made *only on undiluted blood*



o isolat on expe ment negat e
so a n s per me t pos e
DT negat ve
DT t e
CFT neg t ve
CFT t ter
D spo an a y dead
C dead by card ac punc e
S sac f ed

Fig 6

Gr up D 3 guinea pigs inoculated subcutaneously 7 guinea pigs inoculated intra nasally all an mals with 400 000–500 000 toxoplasmas Serum dilutions on log scale

In 5 of 7 intranasally infected animals Toxoplasma was isolated from the blood 3 days after inoculation, that is at the same time as would be expected after subcutaneous inoculation Concerning the du ration of parasitaemia the results agree in the main with those obtained in the preceding group It is obvious even in this small material (Fig 6) that parasitaemia can occur in the presence of antibodies in high titre in the blood (8 58) In one case Toxoplasma could be isolated from the blood 12 weeks after inoculation (10 58)

Group E

The possible appearance of parasitaemia in animals immunized with living parasites was studied in this experiment

In all 18 animals were injected subcutaneously with 5 000 10 000 toxoplasmas Six animals died during the second week after inocula tion The surviving 12 animals were given the same infective dose by the same route at different times between 7 weeks and 8 months later Blood samples for isolation experiments were taken every second or third day during the first 2 weeks after the second inoculation During the experiment 6 guinea pigs died upon cardiac puncture the remain ing 6 animals were sacrified 14 16 weeks after inoculation

From Fig 7 it can be seen that during the experiment Toxoplasma

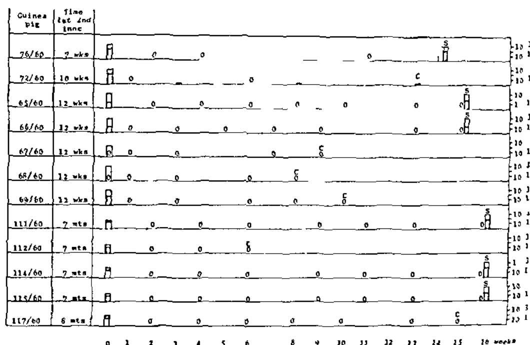


Fig 7

Group F 12 previously immunized guinea pigs inoculated subcutaneously with 5 000–10 000 toxoplasmas Blood and serum dilutions on *log* scale

was isolated from the blood only in 2 cases These two animals had received their first inoculation 7 and 10 weeks earlier, respectively, and both had antibodies in high titre in the blood *It is impossible to know* whether the parasitaemia in these cases is referrable to the first or to the second inoculation

In the remaining guinea-pigs Toxoplasma was not isolated from the blood during the first weeks after inoculation, despite the fact that in several cases, especially those which had been immunized 7 or 8 months earlier, antibody titres had decreased to low values In two cases (67/60, 68/60) the CFT was negative before the second inoculation

Clinical Symptomatology and Death Rate

Fifty-seven of the 78 inoculated animals died during the course of the experiment 33 deaths occurred during or immediately after cardiac puncture the remaining 24 animals died spontaneously There is reason to believe that toxoplasmosis was the cause of death in most of the cases No animal presented signs of any other disease Furthermore, Toxoplasma was isolated from the blood in all of the 18 cases

investigated for parasitaemia Finally the majority, 22 animals, died 10 16 days after inoculation which corresponds to the survival time established in guinea pigs (4) becoming seriously affected

TABLE 1

Death Rate and Signs of Diseases in Guinea Pigs Belonging to the Different Experimental Groups

Group	Number of animals	Number of deaths			Mortality		Number of animales with signes of diseases
		Spontan	Card punct	Sacrificed	Spontan	Card punct	
A	24	5	7	12	20 8	29 1	2
B	9	3	5	1	33 3	55 6	1
C	17	4	11	2	23 5	64 7	3
D	10	6	4		60 0	40 0	8
E	18	6	6	6	33 3	33 3	
Total	78	24	33	21	30 8	42.3	14

Table 1 shows the distribution of deaths in the different groups and the occurrence of clinical signs in the animals Fourteen animals developed visible signs of disease *All of the 7 intranasally infected guinea-pigs* had running noses, cough, and lymphadenopathy of the neck glands Five of these died during the second week after inoculation, the remaining two recovered Eight of the subcutaneously infected [illegible]

[illegible] also observed Five of these animals died and 3 recovered Fourteen animals were *found dead in their cages without* having exhibited signs of disease

It can be seen from table 1 that the mortality from toxoplasmosis was considerably greater in group D than in the other groups Most of the deaths in this group occurred among intranasally infected animals

The total mortality in toxoplasmosis in all of the groups is *20 8* per cent as compared with 32 per cent in an earlier investigation (4) However, the figures must be *considered* as approximate, partly because death occasionally may have been caused by more than one disease and partly since it cannot be precluded that some of the 33 animals which died in connection with heart puncture may have been seriously affected by toxoplasmosis, and hence would have succumbed to its disease

The high rate of deaths ascribable to cardiac puncture (42 3 per cent of the whole material) is of course *regrettable* However, it has been impossible to avoid deaths of animals *so often* deprived of a considerable part of their total blood volume The repeated injuries to the heart muscle may also have played a rôle

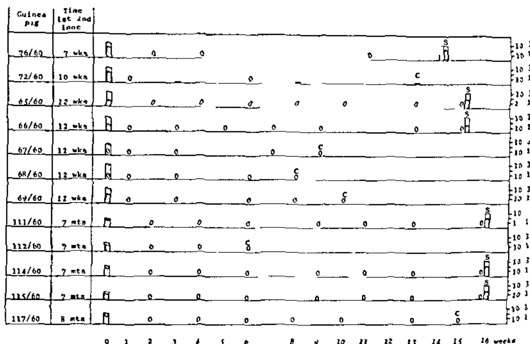


Fig 7

Group F 12 previously immunized guinea pigs inoculated subcutaneously with 5 000–10 000 toxoplasmas Blood and serum dilutions on log scale

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B and parasitaemia developing in animals in group C which were examined less frequently but for a longer period This difference has probably more than one explanation It is to be expected that the parasitaemia gradually decreases with time this would be reflected in the less frequent detection of the parasites in high titre in a more protracted study It is also probable that the considerable loss of blood caused by the frequent cardiac punctures in animals belonging to groups A and B contributes to the occurrence of more pronounced parasitaemia in these animals The fact that all of the 27 investigated animals in groups A and B presented parasitaemia in the second week after infection as apposed to 9 only of 17 animals in group C presenting parasitaemia 2 weeks after inoculation also points in this direction

A phenomenon appearing in several isolation experiments in different experimental groups is that Toxoplasma sometimes was isolated in a higher dilution of blood whereas isolation experiments from lower dilutions of the same blood sample were negative Probably more than one factor is responsible A probable explanation is that Toxoplasma may be present intracellularly in the blood Intracellular parasitaemia with the appearance of Toxoplasma in *e g* elements belonging to the white blood cell system can be expected to give erratic results in the isolations because in such cases more than one parasite is located in a single cell

However the fact which above all points to the occurrence of intracellular parasitaemia is the simultaneous appearance of both Toxoplasma and antibodies in high titre in the same blood sample The transient parasitaemia demonstrated in some animals after 3 weeks is also most easily explained as a result of the occasional appearance in the blood of one or a few Toxoplasma infected cells

Experiment I shows that a thorough Toxoplasma infection give immunity against re induced parasitaemia for at least 8 months This experiment also demonstrates that a real parallelism between DT and CFT titres and immunity does not exist More than one of the immune animals had comparatively low titres This question will be elucidated more closely in a coming investigation

Immunity in toxoplasmosis is complicated and for the most part still obscure As in infections with other intracell lar n -

SUMMARY

T[illegible] Toxo
plas[illegible]
inoc[illegible] were
[illegible] intracutaneously and 7 intranasally
Sixty animals seronegative at the start of the experiments were

Serological investigation

Combining the results from the different groups the conclusions to be drawn are the same as the ones drawn in an earlier investigation on guinea pigs (4). DT proved to be positive at an earlier stage than CFT. One week after inoculation DT was positive in 18 of 29 investigated samples, while CFT was negative in all of the samples. Three weeks after inoculation DT was positive in the dilution 1/250 or above in all of the 16 animals investigated, while CFT remained negative in 11 of 16 examined animals. It was not until the sixth week that all of the investigated animals were positive using CFT, however, and CFT values above 1/120 could not be revealed until 7 weeks after inoculation.

DISCUSSION

The experiments show that parasitaemia regularly appeared in guinea-pigs experimentally (subcutaneously, intracutaneously or intranasally) infected with Toxoplasma if the animals were seronegative before inoculation. Parasitaemia could be demonstrated as early as 3–5 (in one case, 2) days after inoculation, and it persisted for 1–3 (most commonly 2) weeks. After this time transient parasitaemia could be demonstrated in some cases as late as 15 weeks after inoculation.

The results indicate that the parasites do not enter the blood before initial multiplication has taken place. This suggestion is supported by the fact that the period between inoculation and the development of parasitaemia was longer when the infective dose was smaller. Furthermore, the early enlargement of the regional lymph glands in the intranasally infected guinea-pigs points to a primary multiplication of the parasites in the glands. Relevant questions will be elucidated in a coming article.

An attempt was made to estimate the degree of parasitaemia by determining the highest dilution of 0.5 ml of blood (within the limits 1/1–1/1000) causing toxoplasmosis in mice. With the aid of this method a statistically significant continuous increase in the degree of parasitaemia was demonstrated during the first 3–9 days in animals injected with a medium sized infective dose. A similar continuous increase was demonstrated during the first 5–11 days in animals injected with a small infective dose. The gradual increase in parasitaemia supports the concept of a primary local multiplication of the parasites. It is remarkable in this disease that the parasitaemia can persist for as long as 2–3 weeks. No difference was demonstrable in the degrees of parasitaemia to develop in animals with clinical and with asymptomatic toxoplasmosis. It is difficult to give an explanation of this fact. However, it is obvious that the number of circulating parasites is not an adequate characteristic of the severity of the disease.

There is a marked difference in degrees of parasitaemia developing in the more intensively investigated animals belonging to groups A and

FROM THE ENTEROVIRUS DEPARTMENT, STATENS SERUMINSTITUT, COPENHAGEN

COMPLEMENT FIXATION EMPLOYED FOR TYPING OF COXSACKIE A VIRUSES ISOLATED FROM PATIENTS

By

BENTE STAUTZ MUNCH

Received 19 xii 62

In an earlier publication the results of complement fixation (CF) tests with the prototype strains of 24 Coxsackie A and 5 Coxsackie B viruses have been described (1). Fluorocarbon treated torso suspensions from newborn mice were employed as antigens and hyperimmune sera were prepared in adult mice. In the Coxsackie A group, 18 of the 24 types examined gave satisfactory CF and the type specificity of the reacting antigens was found to be excellent. Antigens prepared from the remaining 6 Coxsackie A strains and from the Coxsackie B viruses gave less reproducible results or no CF at all.

The present paper describes the application of CF tests for typing of a number of Coxsackie A strains which has been isolated in newborn mice in this laboratory. Tests with freshly isolated Coxsackie A 1 and B 1 strains have already been described (2).

MATERIALS AND METHODS

Virus strains. A total of 23 Coxsackie A strains were examined comprising six A 1, one A 2, [illegible] een of the stra[illegible] had previously [illegible] out according t[illegible] of CF antigen[illegible] isolated from 10 per cent stool suspensions which had been kept at −20° C since the original isolat[illegible] in newborn mice. 18 out [illegible] which [o]riginally had been [illegible] a C[ox]sackie A 9 strain ha[illegible]

[illegible] of the strains studied were freshly isolated in newborn mice and were first typed in CF tests. Neutralization tests in newborn mice were carried out subsequently.

The help and advice by Dr [illegible] script is gratefully [illegible] by mag. scient. *Hall*[illegible] Mr. *G. Mathiasen* an[illegible] gratefully acknowledged.

[1] Seven of the strains were kindly supplied by Dr. A. *Birkum Petersen* of this institute.

[2] In our experience Coxsackie A 1 strains differ from other Coxsackie A strains by their instability during storage of fecal samples at −20° C.

examined with respect to the occurrence of parasitaemia after inoculation In all animals parasitaemia could be demonstrated

Parasitaemia was demonstrated earliest 3–5 (in one case, 2) days after inoculation When the infective dose was of medium size the parasitaemia appeared *on the third to fourth day*, *when the dose was small*, on the fifth day

Parasitaemia usually continued for 1–3 (most commonly 2) weeks, after which transient parasitaemia could be demonstrated in some cases as late as 15 weeks after inoculation

It could be demonstrated that the degree of parasitaemia increases continuously for 6 days after it was first detected

Toxoplasma organisms and Toxoplasma antibodies in high titre have *been demonstrated in the same blood* sample on *several* occasions *This* observation supports the idea that Toxoplasma can exist intracellularly in the blood

Finally, the investigation shows that a thorough Toxoplasma infection in guinea pigs can produce immunity against the reinduction of parasitaemia for at least 8 months

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TABLE 1

Testing of CF Antigens against 4 Pools of Coxsackie A Hyperimmune Sera

Antigen		Hyperimmune serum pools*								Normal serum		Antig control	CF reaction with pool No	Result of neutral tests	ID of CF antigen neg log
		I 1-5		II 6-8 10		III 11-15		IV 16-19							
No	Dilutions	1*	6§	1	6	1	6	1	6	5	30				
3088	1§	1	0	+	0	+	0	+	0	0	0	+	0 (I n s†)	A 16	3.5
	2	1	0	0	0	0	0	0	0	0	0	0			
3171	1	3	4	+	0	+	0	+	0	0	0	+	I	A 4	ca 7.1
	2	3	3	0	0	0	0	0	0	0	0	0			
3420	1	1	+	+	0	+	0	3	2	0	0	0	IV (I n s)	A 16	4.9
	2	1	0	0	0	0	0	7	2	0	0	0			
3614	1	1	+	3	3	+	0	+	0	0	0	0	II (I n s)	A 8	7.1
	2	1	0	3	3	0	0	0	0	0	0	0			
4353	1	3	1	+	0	1	0	+	0	0	0	0	I III	A 5	7.8
	2	3	1	0	0	1	0	0	0	0	0	0			
4617	1	3	1	+	0	+	0	+	0	0	0	0	I	A 1	ca 6.8
	2	1	+	0	0	0	0	0	0	0	0	0			
5317	1	1	+	3	4	+	0	+	0	0	0	+	II (I n s)	A 10	6.6
	2	1	0	2	2	0	0	0	0	0	0	0			
5432	1	3	3	+	0	+	0	+	0	0	0	0	I	A 1	ca 6.7
	2	3	3	0	0	0	0	0	0	0	0	0			
Serum control		+	0	0	0	0	0	0	0	0	0				

* Dilutions of individual sera in all pools 1 5 and 1 30
§ Reciprocal of serum or antigen dilutions (1 = undiluted 2 = 1:2, 6 = 1:6, etc.)
† n s — non-specific
Other legends see text

Simultaneous complement titration in Veronal buffer

Units of complement	2	1.5	1	0.75	0.5
Fixation grade	0	0	(+)	+	1

Preparation of CF antigen The method of antigen preparation has been presented in detail in preceding papers (1, 2) and shall only briefly be reviewed here Two litters of newborn mice usually less than 24 hours of age were inoculated intraperitoneally with 0 02 ml of a 10 per cent stool suspension to which penicillin and streptomycin had been added The animals were observed daily for 14 days for typical signs of illness and diseased mice were killed by ether

In order to increase the virus titer, one additional mouse passage of the virus was routinely carried out in two litters of newborn mice using a 10 per cent torso suspension as passage material All diseased animals were killed by ether and stored at —20° C For antigen preparation a 20 per cent suspension of the torsos was prepared and purified with 'Freon 112, followed by addition of guinea pig serum and inactivation The antigens were stored at —20° C and centrifuged before use

Virus titrations were performed in newborn mice as described earlier (1)

Complement fixation tests All details have been given in earlier papers (1 2)

Since the Coxsackie viruses A 20 through 24 were not available to us until late in this study, testing of the CF antigens against hyperimmune sera of these virus types were not included

EXPERIMENTAL AND RESULTS

Typing of "unknown" antigens was performed in two steps First the antigens were examined in CF tests against pools of hyperimmune mouse sera Whenever an antigen was found to react with one of the serum pools, the next step was to test the antigen against the individual sera contained in the pool

It was studied whether typing of antigens could always be done employing antigens undiluted or diluted 1 2 Undiluted antigens were used to ensure maximal amounts of antigen in the test, in case the potency of preparation was low A 1 2 dilution was included with the possibility in mind that a concentrated antigen may give rise to a prozone phenomenon, or possibly to non-specific reactions

In order to simplify the tests, and to save antigens and sera, titrations of complement in the presence of the dilutions of antigens and sera employed in the tests proper were as a rule omitted This was found justifiable since experience gained in numerous previous experiments had shown that anticomplementary activity of the kind of antigen and antisera preparations employed was so slight that it did not interfere with the reading of the results (1) A control titration of complement in veronal buffer was always included in the experiments

As regards reading of the CF tests, fixation grades of 3 or 4 with one or more of the antigen- and serum dilutions examined were recorded as positive reactions Whether fixation grades of 2 or less were recorded as specific was determined by comparison with the results obtained with the other serum types and with the controls included in the tests If equivocal reactions were obtained with an antigen, the test was repeated, and if the results were still equivocal it was attempted to prepare a more potent CF antigen using third mouse passage material for the preparation

CF typing experiments employing low dilutions of antisera When the first experiments in this study were performed, the exact CF titers of all hyperimmune sera had not yet been determined Serum pools—each

containing equal amounts of 5 different types of hyperimmune sera—were therefore used undiluted and diluted 1 6 this representing dilutions of 1 5 and 1 30 respectively of the individual sera in the pools In some of the pools normal mouse serum was added to obtain these dilutions The same two dilutions *i e 1 5 and 1 30 of the individual* sera were used during the second step of the experiments

In all the tables in this paper the results of typing of the virus isolates by neutralization tests in newborn mice are listed for comparison with the CF results The infectious titers of the antigens have also been recorded These data will de discussed later in the paper

In Table 1 results obtained with 8 antigens in the first step of a CF typing experiment are recorded It will be seen that all the antigens except No 3088 gave a positive reaction with the pool containing the homologous hyperimmune serum and with this pool only A slight fixation was encountered between all the undiluted serum pools and all the *undiluted antigens this being especially pronounced for pool I* which also gave weak reactions with antigens diluted 1 2 However, experience from previous experiments (1) allowed the interpretation of these reactions as being non specific

A slight crossing was found between antigen No 4353 and serum pool III containing hyperimmune sera A 11 through 15 Since this antigen had previously been typed in neutralization tests as a Coxsackie A 5 *strain* this reaction could be reasonably ascribed to the cross reactions known to exist between types A 5 and A 12 (1)

Table 2 presents the results *obtained* when the 7 reacting antigens from Table 1 were tested against the individual sera in the reacting pools As will be seen each CF antigen was found to react with the homologous serum only

TABLF 3

Testing of Coxsackie A CF Antigens Prepared from Second and Third Mouse Passage Material

Antigen No	Dilut	Hyperimmune serum pools I 1-5		II 6, 8-10		III 11-15		IV 16-19		Ag control	CF reacti with pool No	Result of neutral tests	ID_{50} of CF antigen neg log
		1*	6*	1	6	1	6	1	6	–			
5913 2 pass	1	2	1	0	0	0	0	0	0	0	I ?	A 1	ca 6 6
	2	1	+	0	0	0	0	0	0	0			
5913 3 3 pass	1	3	3	0	0	0	0	0	0	0	I	A 1	7 2
	2	3	3	0	0	0	0	0	0	0			
Serum control		0	0	0	0	0	0	0	0	0			

Reciprocal of serum or antigen dilutions
Legends see Table 1

Simultaneous complement titration in Veronal buffer

Units of complement	2	1 5	1	0 75	0 5
Fixation grade	0	0	0	+	+

TABLE 2

Testing of CF Antigens from Table 1 against Individual Sera Contained in the Reacting Serum Pools

Antigen		Coxsackie A hyperimmune sera													Antig control	Result of CF tests	Result of neutral tests	ID_{50} of CF antigen neg log
		Pool I					Pool II				Pool IV							
		A 1	A 2	A 3	A 4	A 5	A 6	A 7	A 8	A 10	A 16	A 17	A 18	A 19				
No	Dilut	5* 30	5 30	5 30	5 30	5 30	5 30	5 30	5 30	5 30	5 30	5 30	5 30	5 30				
3171	1*	0 0	0 0	0 0	4 4	0 0	— —	— —	— —	— —	— —	— —	— —	— —	0	A 4	A 4	ca 7.1
	2	0 0	0 0	0 0	4 4	0 0	—		— —	— —	—	— —	— —	— —	0			
3420	1		—	— —		— —	— —	— —	— —	— —	4 3	0 0	0 0	0 0	0	A 16	A 16	4.9
	2	— —	— —	—	—	— —	— —	— —	— —	— —	3 3	0 0	0 0	0 0	0			
3614	1	—	—	—		— —	0 0	0 0	4 4	0 0	— —	— —	— —	— —	0	A 8	A 8	7.1
	2	— —	—	—		— —	0 0	0 0	4 4	0 0	—	—	— —	— —	0			
4353	1	0 0	0 0	0 0	0 0	4 4	— —	— —	— —		—	— —	— —	— —	0	A 5	A 5	7.8
	2	0 0	0 0	0 0	0 0	4 4	— —	— —	— —		— —	— —	— —	— —				
4617	1	3 2	0 0	0 0	0 0	0 0		—		—	—	—	—	—	0	A 1	A 1	ca 6.8
	2	1 2	0 0	0 0	0 0	0 0	—			—	—	—	—	—	0			
5227	1			—	—	— —	0 0	0 0	0 0	3 3	—	—	—	— —	0	A 10	A 10	6.6
	2		—		—	—	0 0	0 0	0 0	3 4	—	—		— —	0			
5432	1	4 4	0 0	0 0	0 0	0 0		— —	— —			—	—	— —	0	A 1	A 1	ca 6.7
	2	3 3	0 0	0 0	0 0	0 0	—		—	—	—	—	— —	— —	0			
Serum control		0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0			

— = not done
* Reciprocal of serum or antigen dilutions

Simultaneous complement titration in Veronal buffer

Units of complement	2	1.5	1	0.75	0.5
Fixation grade	0	0	+	+	1

containing equal amounts of 5 different types of hyperimmune sera—were therefore used undiluted and diluted 1 6 this representing dilutions of 1 5 and 1 30 respectively, of the individual sera in the pools In some of the pools normal mouse serum was added to obtain these dilutions The same two dilutions, i e 1 5 and 1 30, of the individual sera were used during the second step of the experiments

In all the tables in this paper the results of typing of the virus isolates by neutralization tests in newborn mice are listed for comparison with the CF results The infectious titers of the antigens have also been recorded These data will de discussed later in the paper

In Table 1 results obtained with 8 antigens in the first step of a CF typing experiment are recorded It will be seen that all the antigens except No 3088 gave a positive reaction with the pool containing the homologous hyperimmune serum and with this pool only A slight fixation was encountered between all the undiluted serum pools and all the undiluted antigens, this being especially pronounced for pool I which also gave weak reactions with antigens diluted 1 2 However, experience from previous experiments (1) allowed the interpretation of these reactions as being non specific

A slight crossing was found between antigen No 4353 and serum pool III containing hyperimmune sera A 11 through 15 Since this antigen had previously been typed in neutralization tests as a Coxsackie A 5 strain this reaction could be reasonably ascribed to the cross reactions known to exist between types A 5 and A 12 (1)

Table 2 presents the results obtained when the 7 reacting antigens from Table 1 were tested against the individual sera in the reacting pools As will be seen each CF antigen was found to react with the homologous serum only

TABLF 3

Testing of Coxsackie A CF Antigens Prepared from Second and Third Mouse Passage Material

Antigen No	Dilut	Hyperimmune serum pools I 1 5 1*	I 1 5 6*	II 6 8 10 1	II 6 8 10 6	III 11 15 1	III 11 15 6	IV 16-19 1	IV 16-19 6	Act'g control	CF reacting with pool No	Result of neutral tests	ID_{50} of CF antigen neg log
5913 2 pass	1	2	1	0	0	0	0	0	0	0	I ?	A 1	ca 6 6
	2	1	+	0	0	0	0	0	0	0			
5913 3 3 pass	1	3	3	0	0	0	0	0	0	0	I	A 1	7 2
	2	3	3	0	0	0	0	0	0	0			
Serum control		0	0	0	0	0	0	0	0	0			

Reciprocal of serum or antigen dilutions
Legends see Table 1

Simultaneous complement titration in Veronal buffer

Units of complement	2	1 5	1	0 75	0 5
Fixation grade	0	0	0	+	+

TABLE 4

Testing of CF Antigens from Table 3 against Individual Coxsackie A Antisera Containing in the Reacting Serum Pool

Antigen No	Dilut	Hyperimmune serum pool I: A 1, 5*	A 1, 30	A 2, 5	A 2, 30	A 3, 5	A 3, 30	A 4, 5	A 4, 30	A 5, 5	A 5, 30	Antig control	Result of CF tests
5913, 2 pass	1*	3	3	0	0	0	0	0	0	0	0	0	A 1
	2	2	1	0	0	0	0	0	0	0	0	0	
5913 3 3 pass	1	4	4	0	0	0	0	0	0	0	0	0	A 1
	2	4	4	0	0	0	0	0	0	0	0	0	
Serum control	–	0	0	0	0	0	0	0	0	0	0	0	

* Reciprocal of serum or antigen dilutions

Simultaneous complement titration in Veronal buffer

Units of complement	2	1 5	1	0 75	0 5
Fixation grade	0	0	+	1	1

TABLE 5

Testing of CF Antigens against 4 Pools of Coxsackie A Hyperimmune Sera

Exp No	Antigen No	Dilut	Hyperimmune serum pools*: I	II	III	IV	Antig control	CF reaction with pool No	Result of neutral tests	ID$_{50}$ of CF antigen neg log
348	6706	1§	4	0	1	0	+	I III	A 5	7 9
		2	3	0	1	0	0			
	6725	1	3	0	1	0	+	I	A 2	7 0
		2	2	0	0	0	0			
	Control	1	1	0	+	0	0			
		2	+	0	0	0	0			
357 A	6845	1	2	0	0	0	+	I	A 1	ca 6 0
		2	1	0	0	0	0			
357 B	6934	1	+	0	0	3	+	IV	A 16	5 5
		2	+	0	0	2	0			
387	7119	1	0	0	0	1	0	IV ?	A 16	5 5
		2	0	0	0	+	0			

** 1 20

*

§

A1

Simultaneous complement titrations in Veronal buffer

Exp No	Units of complement: 2	1 5	1	0 75	0 5
348	0	0	0	0	(+)
357 A	+	+	+	+	+
357 B	0	0	0	0	+
387	0	0	0	0	0

TABLE 6

Testing of CF Antigens from Table 5 against Individual Sera Contained in the Reacting Serum Pools

Exp No	Antigen		Coxsackie A hyperimmune sera														Antig control	Result of CF test	Result of neutral tests	ID50 of CF antigen neg log
			Pool I					Pool III					Pool IV							
	No	Dilut	A 1 16*	A 2 64	A 3 128	A 4 128	A 5 128	A 11 16	A 12 128	A 13 16	A 14 128	A 15 32	A 16 32	A 17 16	A 18 16	A 19 16				
358	6706	1*	0	+	+	+	4	0	1	0	0	0					+	A 5	A 5	7 9
		2	0	0	(+)	(+)	4	0	1	0	0	0	–			–	0			
	6725	1	1	4	1	1	1					–		–		–	0	A 2	A 2	7 0
		2	0	3	(+)	(+)	(+)										0			
	6845	1	2	+	+	+	+	–	–	–		–	–	–			+	A 1	A 1	ca 6 0
		2	+	+	+	+	+					–		–		–	0			
	6954	1								–			3	0	0	0	+	A 16	A 16	5 5
		2								–		–	2	0	0	0	0			
	Serum control		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
388	7119	1		–	–	–			–		–		3	0	0	0	0	A 16	A 16	5 5
		2		–	–	–			–	–			2	0	0	0	0			

– — not done

* Reciprocal of serum or antigen dilutions

Simultaneous complement titrations in Veronal buffer

Exp No	Units of complement				
	2	1 5	1	0 75	0 5
358	0	0	0	0	+
388	0	0	0	+	+

TABLE 7

Summary of Complement Fixation Tests with Antigens Prepared from Second Mouse Passage of Coxsackie A Virus Strains Recovered from Stool Specimens

Type* of antigen	Number of antigens exam	Number of antigens typed	Antigens not reacting	ID_{50} of reacting antigens neg log	ID_{50} of non reacting antigens neg log
Coxs A 1	6	5	1	6 0 6 8	4 9
– A 2	1	1	0	7 0	
– A 4	2	2	0	7 1–8 0	
– A 5	5	5	0	7 5 7 9	
– A 8	3	3	0	7 0 7 6	
– A 10	1	1	0	6 6	
– A 16	5	4	1	4 9–5 5	3 5
Total	23	23	2		

* Type as determined by neutralization tests in newborn mice

Tables 3 and 4 show the difference in reactions obtained when a weak antigen No 5913 prepared from second mouse passage material was compared to the more potent antigen, No 5913-3, prepared from third mouse passage of the virus strain It can be seen that although the reactions encountered between serum pool I and antigen No 5913 were not much stronger than the non-specific reactions occasionally obtained with this serum pool, they were in this case nevertheless specific Table 4 shows that stronger reactions were obtained in the second step of the typing The control titrations of complement indicate that variations in the amount of complement present in the tests account for these differences in fixation grades

CF typing experiments employing higher dilutions of antisera When later in this study the CF antibody titers of all the types of hyperimmune sera had been determined, it was attempted to employ higher serum dilutions in order to save sera and to avoid non-specific reactions occasionally seen with more concentrated sera Depending on the 'average' level of the CF titers of the individual sera, the final dilution of all the sera in the pools was chosen to be either 1 40 or 1 20 Hyperimmune sera of the Coxsackie types giving "unreliable" CF reactions with the prototype antigens (1) were however, always employed in a dilution 1 16

In Tables 5 and 6 are presented results obtained with 5 CF antigens when these higher serum dilutions were employed It will be seen that even here slight non specific reactions were now and then encountered, but otherwise full agreement was obtained between typing by CF tests and by neutralization tests in newborn mice

It was investigated whether employing the rather poor antigen No 6845 in a volume of 0 2 ml instead of the usual 0 1 ml would improve the results It was found that 0 2 ml gave slightly stronger reactions with pool I than did 0 1 ml, but the titrations of complement showed

that the application of a double amount of antigen also resulted in an appreciable increase of its anticomplementary effect

Table 7 presents a summary of the CF typing of all the 23 stool strains of Coxsackie virus included in the study In 21 out of the 23 samples examined agreement between the neutralization tests and the CF tests was obtained when second mouse passage material was used for CF antigen preparation

In the two instances where second mouse passage did not provide a sufficiently potent CF antigen the experiments were repeated using antigens prepared from third mouse passage material Typing by the CF method was now succesful with both strains one A 16 strain and one A 1 strain respectively

DISCUSSION

In a preceding study (1) it was found that satisfactory CF antigens could be prepared from the prototype strains of 18 out of 24 prototype Coxsackie A strains while 6 prototype antigens (types 11, 13 17 18 19 and 21) gave negative or unreproducible results

The present experiments have shown that the CF technique could be employed as routine method for typing of 23 Coxsackie A virus strains freshly isolated in newborn mice which represented all the Coxsackie A virus isolated—other than type 9[3]—available in the laboratory at the time of the experiments Satisfactory CF antigens were thus prepared from second mouse passage of 21 out of the 23 Coxsackie A strains One A 1 strain and one A 16 strain required a third passage in mice before yielding a good antigen None of the 23 strains belonged to the difficult 6 Coxsackie A types listed in the beginning of this chapter

Since Coxsackie B viruses do not usually yield satisfactory CF antigens when mouse tissue material is employed for the preparation (1 [illegible]) tissue culture antigen is preferable for CF typing of the Coxsackie B types and no further attempts have been made in our laboratory with mouse tissue CF antigens of the Coxsackie B strains

From earlier experiments it was concluded that only mouse tissue suspensions with a reasonably high infectious titer would consistently yield reliable CF antigens (1) This observation has been confirmed by the results of infectivity titrations of the present CF antigens It will thus be seen from the tables that CF antigens prepared from high titer Coxsackie A virus strains showed good CF potency and were all [illegible]

[illegible] antigen showed low or no CF potency When third mouse passage of these virus strains was used for antigen preparation they both gave reliable although weak

[3] For CF typing of Coxsackie A 9 virus strains tissue culture antigens have ordinarily been employed in this laboratory (4)

fixation with the homologous sera The infectious titers of these 3' passage antigens were ca $10^{-5.9}$ and $10^{-5.6}$, respectively

Whether the infected material from the suckling mice will yield a satisfactory CF antigen can to some extent be predicted from the ratio of mice succumbing to infection and from the character and degree of paralysis, and from the incubation time It has been our experience that the majority of the satisfactory antigens was obtained when definite signs of illness was observed in most of the mice on the second or third day after inoculation

The bacterial content in an inoculum may occasionally give signs of illness in the mice resembling those produced by Coxsackie viruses In such instances ether treatment of the inoculum and addition of adequate antibiotics have proved useful

The exact amount of complement actually present in the various test tubes is one of the important factors in CF typing experiments This is illustrated by the difference in fixation grades observed in the first and second type experiments with the antigens Nos 5913 and 5913-3 (Tables 3 and 4) and with antigen No 7119 (Tables 5 and 6) When weak antigens are employed it would in some instances undoubtedly be useful if several dilutions of complement were included in the same experiment (5)

In the present study slight non-specific reactions were occasionally encountered when the sera were used in pools even in the dilutions 1 20 and 1 40 Since these reactions never were stronger than + to 1 reactions, they were, however, not difficult to distinguish from specific reactions The non-specific reactions were found more often when the sera had been stored at +4° C instead of at —20° C before use in CF tests To avoid non-specific reactions it seems generally advisable to include the individual sera in the pools in a dilution four to eigth times lower than the CF antibody titer of the serum, even if this will make the preparation of the serum pools slightly more elaborous (1) However, in certain instances, particularly when antigens expected to be of low potency are to be tested, the use of more concentrated sera may still be preferable

The number of freshly isolated strains examined in this study has been relatively small However, the data from this and preceding studies (1, 2) show that although it may be necessary to resort to typing by neutralization tests in newborn mice of certain Coxsackie A strains, the described CF method may be expected to be a valuable tool in routine typing of the majority of freshly isolated Coxsackie A virus strains

SUMMARY

In the present study Coxsackie A virus strains isolated from patients by inoculation of newborn mice have been typed by the complement fixation technique

The CF antigens were prepared from mouse torso tissue employing treatment with trochlorotrifluoroethane ("Freon 112"), and guinea pig serum was subsequently added (1, 2)

The 23 Coxsackie A virus strains examined comprised six A 1, one A 2, two A 4, five A 5, three A 8, one A 10, and five A 16 strains Satisfactory CF antigens were prepared from second mouse passage of 21 out of 23 of the freshly isolated strains One Coxsackie A 1 strain and one Coxsackie A 16 strain could be typed after one additional mouse pasage

Various technical problems concerning the practical use of the CF method for routine typing of Coxsackie A virus strains freshly isolated in newborn mice are discussed

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FROM THE UNIVERSITY OF BERGEN, SCHOOL OF MEDICINE THE GADE INSTITUTE, DEPARTMENT OF MICROBIOLOGY, BERGEN, NORWAY

IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDES FROM STAPHYLOCOCCUS EPIDERMIDIS

1. *Isolation and Chemical Characterization*

By

NORVALD LOSNEGARD and PER OEDING

Received 4.1.63

On the basis of the precipitation test *Julianelle & Wieghard* (26) have divided staphylococci into pathogenic type A and nonpathogenic type B strains containing the corresponding carbohydrates A and B. Later *Thompson & Khorazo* (36) described three new groups, C, BC, and O, following precipitation of nonpathogenic strains. *Staph. aureus* strains lacking carbohydrate A were placed into another group by *Cowan* (6). Carbohydrates A and B have to some extent been characterized chemically by *Wieghard & Julianelle* (37). Significant differences in optical rotation, fermentable and nonfermentable sugars were found. Detailed chemical and serological examinations of polysaccharide A from two *Staph. aureus* strains have been reported by *Haukenes* (14–20). Polysaccharide A was found to be serologically identical and chemically very similar to teichoic acid from the wall of *Staph. aureus* (21).

To our knowledge there have been no further reports on the chemical composition of the group-specific polysaccharides present in *Staph. epidermidis*.

MATERIALS AND METHODS

Strains. Four *Staph. epidermidis* strains were used in the present experiments. Strain 1254 was isolated from the nose, 1268 from pus, and 1622 and 3519 from urine. All the strains were negative on coagulase, mannitol, hyaluronidase, phosphatase, and esterase test. The strains used by *Julianelle & Wieghard* (26) and *Thompson & Khorazo* (36) were not available.

Growth and harvesting. Eighteen hour cultures of the strains, grown on nutrient agar in Petri dishes of 14 cm diameter, were harvested by scraping with a glass rod.

Disruption of cells was obtained in a Hughes bacteria press at —25° C (25).

Extraction. The extraction procedure described by *Haukenes et al.* (22) was adopted with some modifications. The disrupted cells were suspended in distilled water, stirred and centrifuged. The supernatant was adjusted to pH 4.2 with 0.1 N HCl and the precipitate removed. After raising the pH to 6.9 with 0.1 N NaOH the supernatant was used for further extraction of the cells. This process was repeated 10 times. On the two last occasions the microbes were resuspended in distilled water and the pH brought to 4.2 before centrifugation. Extraction and centrifugation were performed in the cold except for strain 3519 which was extracted in an incubator at 37° C.

Ion exchange columns were used for further purification of the polysaccharide material

Diethylaminoethyl (DEAE) cellulose 100–230 mesh was prepared as described by *Peterson & Sober* (31) The procedure for running the column was principally that described by *Haukenes* (14) The system was arranged to give a continuous increase of the KCl gradient from 0 to 0 4 M

De-Acidite FF columns The resin (The Permutit Co London) of 100–200 mesh and 3 5 per cent cross linkage was suspended in 80 per cent formic acid and supplied to the column More 80 per cent formic acid was run through then water 3 25 M ammonium formate of pH 5 2 and finally water Both stepwise and continuous increase in the ammonium formate gradient up to a molarity of 3 0 were tried

Amberlite IR 120 The resin (Rohm & Haas Co Philadelphia) 100–230 mesh 8 per cent cross linkage was employed to fractionate sugars amino sugars and amino acids in the hydrolysate of the polysaccharide material purified on the foregoing columns Stepwise elution with 0 33 N HCl (7) 1 5 N HCl 2 5 N HCl and 4 N HCl (35) was used

Dialysis was carried out in cellophane tubes (Kalle & Co Wiesbaden)

Sephadex G 25 and Sephadex G 50 (Pharmacia Uppsala) columns were used for gel filtration (14)

Hydrolysis was performed in 3 N HCl for 3 hours at 100° C in glass stoppered tubes

Paper chromatography The fractions from the Amberlite IR 120 columns were pooled according to the preliminary tests for sugars amino sugars and amino acids The batches were evaporated to dryness under reduced pressure Descending chromatograms on Whatman No 1 paper as supplied were run in the following systems

A Phenol water (4 1 w/v) (4) The phenol was purified by distillation with

cent ammonia on the bottom of

(1 modified)

F Methanol water 10 N HCl pyridine (80 17 5 2 5 10) (32)

Spray reagents

a Ninhydrin 0 1 per cent in water saturated n buthanol (4) or in acetone (24)

amino sugars on paper chro

Paper ele

Analytical methods

Hexosamines were determined by the method of *Rondle & Morgan* (33)

For *carbohydrates* the quantitative Molisch test described by *Dische* (8) was employed

Relative viscosity was determined in an *Ostwald* viscometer (16) on aqueous solutions of materials at a concentration of 5 mg ml

Specific rotation was measured in a Zeiss Circle Polarimeter (16)

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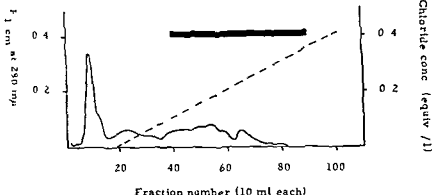
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Fraction number (10 ml each)

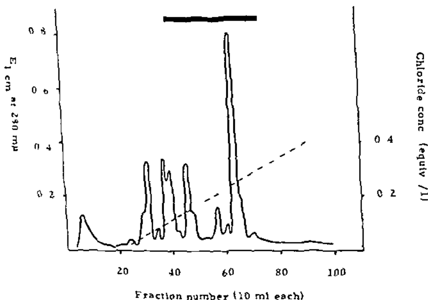
Fig 1

Chromatography of polysaccharide 1254 on DEAE cellulose

—— $E_{1\,cm}$ at 280 mμ

– – – Chloride concentration

▬ Major part of polysaccharide material



Fraction number (10 ml each)

Fig 2

Chromatography of polysaccharide 1622 on DEAE cellulose

—— $E_{1\,cm}$ at 280 mμ

– – – Chloride concentration

▬ Major part of polysaccharide material

Deoxypentoses and *pentoses* were examined by *Dische's* diphenylamine test (9) and *Bial's* test (2) respectively

The qualitative *Molisch test* for carbohydrates and the qualitative *biuret test* for proteins were carried out as described in (22)

Precipitation by ring test was performed by the method used by *Haukenes et al* (22)

Agar precipitation, as used by *Haukenes & Oeding* (23), was used for the examination of polysaccharides during the purification steps

The *immune sera* used in the precipitation tests were produced by intravenous injections of formalin-killed bacteria as described by *Oeding* (28)

RESULTS

It was observed that if the suspensions of strains 1254, 1268, and 1622 were placed in an icebox for 2–4 weeks after the first series of extractions had been completed, large amounts of serologically active material were released In strains 1268 and 1622 this material formed the major part of the total active material obtained Further extractions gave only negligible amounts The first extraction series of strain 3519 included also repeated extraction at 37° C for 2–3 days In contrast to the above observation strain 3519 appeared to be nearly emptied of active material during the first extraction period, as further extraction in an icebox, followed by extraction at 37° C gave but little active material This is in accordance with the observation by *Haukenes* (20) that polysaccharide A is released from the cell wall of *Staph aureus* by a lytic enzyme

The polysaccharides showed rapid migration on agar precipitation On dialysis serologically active material passed through the cellophane membrane very slowly The loss of material on dialysis over night, as calculated from ring test precipitation, was about 0 5 per cent

The yield of purified polysaccharides varied from about 5 to about 12 mg per g wet weight of bacteria

Ion Exchange Chromatography

Polysaccharide 1268 produced a curve similar to that of polysaccharide 1254 (Fig 1), whereas the 3519 preparation behaved like 1622 (Fig 2) in the cellulose column Some ultraviolet light absorbing material goes straight through the columns Serologically active material is mainly excluded in the region 0 1–0 35 M KCl, as examined by the ring test The De-Acidite FF columns also excluded ultraviolet light absorbing material at the beginning of the run, whereas active material was liberated from just below 1 M to 3 M ammonium formate Ultraviolet light absorption, obviously not associated with the serologically active material, also occurs in the region of the active material

Ultraviolet light absorbing fractions were removed as far as possible by ion exchange chromatography without loosing too much of the active material During the purification steps the material showed increasing ring test titre and diminishing ultraviolet light absorption The

sence of glucosamine Amino sugar test was carried out on the 1268 hydrolysate fractions, measuring the extinction at 530 mμ, immediately and after 1½ hours, and at 505 mμ after 24 hours The last 3 positive fractions of a total of 10, showed markedly higher extinction values at 505 mμ than at 530 mμ, thus behaving like muramic acid (7) Corresponding examinations of the other polysaccharide hydrolysates were not performed The amino acids, as pointed out by paper chromatography, were excluded in the order reported by *Stein & Moore* (35)

Paper Chromatography

While system A indicated the presence of lysine in the polysaccharide preparations from all the strains, there were some difficulties in detecting lysine in system B as the paper was discoloured in the appropriate region The material was therefore run in system E and sprayed with 0 1 per cent ninhydrin in acetone, and the characteristic purple colour, which changed to brown, developed Also the distance travelled was in good agreement with that for the lysine reference This chromatogram also excluded the presence of $\alpha\varepsilon$-diaminopimelic acid, which, besides, has not been found in staphylococcal cell walls (34) One more chromatogram was run in system E, the spots eluted and run anew in system B and lysine was found to be present Glutamic acid, glycine, alanine and serine in addition to lysine, were found in the polysaccharide preparations from all the strains

The pooled fractions containing amino sugars gave 2 well separated spots with the Flson-Morgan reagents both in system A and B Ninhydrin oxidized (12) polysaccharide hydrolysates from all the strains gave spots corresponding to arabinose in system A and C, showing the major amino sugar component to be glucosamine The R_G values of the other component were in good agreement with those for muramic acid found by *Crumpton* (7)

The Molisch positive materials from the Amberlite IR-120 columns were run in system A and spots with R_F 0 35 0 37 were eluted and used for running in system C and for electrophoresis Glucose seemed to be present in every one of the preparations

Samples of 1 2 mg polysaccharide material were hydrolyzed, evaporated to dryness and examined for sugar alcohols in system D As references were used glycerol and hydrolyzed samples from a ribitol teichoic acid and a glycerol teichoic acid The teichoic acids were kindly furnished by Professor J Baddiley Newcastle Glycerol was found in the strain 1254 and 1268 material whereas ribitol and anhydroribitol were found in the preparations from 1622 and 3519

Small amounts of Elson-Morgan reacting material, assumed to be unhydrolyzed residues were detected on paper chromatograms These substances were eluted prior to the amino sugars on the Amberlite IR 120 columns

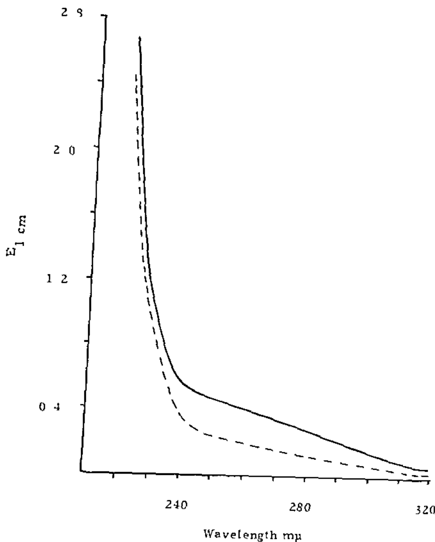


Fig 3

Ultraviolet absorption spectra of polysaccharide preparations from strains 1254 (- -) and 1622 (——) both in a concentration of 5 mg/ml in aqueous solution

ultraviolet absorption spectra of polysaccharides 1254 and 1622 are shown in Fig 3

Molisch positive material from 25 mg hydrolyzed polysaccharide samples went straight through the Amberlite IR-120 columns, the test being strongly positive on the samples from strains 1254 and 1268, weakly positive on those from strains 1622 and 3519 Amino sugars started to go out at a volume of about 70 ml and were completely excluded at about 100 ml on elution with 0 33 N HCl The volume from the start to the fraction which contained the greatest amount of amino sugars was compared with the corresponding volumes for glucosamine and galactosamine references For glucosamine the volume was 86 ml, for galactosamine 92 ml For the amino sugars from the 4 polysaccharides the volumes varied from 85 to 87 ml, thus indicating the pre-

Analyses of Polysaccharides

Table 2 comprises all the data obtained from the quantitative as well as the qualitative analyses of the polysaccharide preparations The nitrogen content is low in each of the polysaccharides, whereas the reducing sugars, calculated as glucose, and the phosphorus content are high Inorganic phosphorus was not present The hexosamine content of polysaccharides 1622 and 3519 is markedly higher than that of the polysaccharides 1254 and 1268 However, the most significant differences between polysaccharides 1254 and 1268, on the one hand, and those of 1622 and 3519 on the other are the neutral sugar content, the specific rotation and the presence of glycerol in the former group and ribitol in the latter

The amino acids alanine, glycine, glutamic acid, lysine, and serine were demonstrated in each of the polysaccharides Visually glycine and alanine seemed to be present in large amounts, whereas the content of serine was small

DISCUSSION

Crude polysaccharide 1622 (and 3519) seems to be more contaminated with ultraviolet light absorbing materials than crude polysaccharide 1254 (and 1268), as the former, in contrast to the latter, produces several distinct peaks (Fig 1 and 2)

The finding that the activity increased with decreasing ultraviolet light absorption during purification of the materials indicates that absorption is not associated with the serologically active material There is however still some ultraviolet light absorbing material present in the purified polysaccharides (Fig 3) None of them produced the nucleic acid peak at 260 mμ, nor the protein peak at 280 mμ, which is in accordance with the negative Bial and Dische tests (Table 2), the low nitrogen content found on analysis, and the lack of aromatic amino acids in acid hydrolysates It is believed that re-chromatography on columns together with an even more drastic removal of ultraviolet light absorbing fractions would have rendered the polysaccharides almost free of ultraviolet light absorbing material One reservation has to be made As will be reported in a forthcoming article, the preparations may be mixtures of more antigens and the weaker ones may possess ultraviolet light absorbing ability

All the polysaccharides were excluded on the *Sephadex G 50 column*, suggesting that the molecular weights were above 8 000 to 10,000 According to the low relative viscosity the partial dialyzability and the rapid migration in agar the molecular weights can, however, not be very high These statements are valid for polysaccharide A too, as reported by *Haukenes* (16)

The difference in carbohydrate content of polysaccharides 1622 and 3519 as compared to polysaccharides 1254 and 1268 must be due to a

Electrophoresis

The paper chromatograms indicated that glucose was present Verification of this by electrophoresis was only partly successful

Only traces of a sugar, that behaved as glucose, were found in polysaccharide 3519 Polysaccharide 1622 contained a sugar that behaved like glucose at pH 9 2 and like galactose at pH 8 6 The converse was the case for the sugar from polysaccharide 1254 These findings have to be checked with new portions of material The results obtained with the sugar from polysaccharide 1268 are listed in Table 1 The sugar is most probably glucose

TABLE 1

Mobilities on Paper Electrophoresis of the Sugar from Polysaccharide 1268 and Reference Sugars in Borate at various pH Values, on Whatman No 1 Paper

Sugar	*Distance from origin cm*		
	pH 9 2	pH 8 6	pH 7 0
Polysaccharide 1268 sugar	11 4	11 5	9 8
Mannose	7 2	5 7	
Arabinose	11 5	12 3	
Glucose	*11 5*	*11 3*	9 5
Galactose	10 8	9 9	10 0

TABLE 2

Analytical Data

	Polysaccharide from Staph epidermidis strain			
	1254	1268	1622	3519
Nitrogen, per cent	2 34	3 43	2 93	3 20
Phosphorus per cent	7 72	6 98	6 76	7 20
Reducing sugars (as glucose) per cent	42 16	46 63	40 80	40 80
Hexosamines (as glucosamine) per cent	13 69	16 45	23 34	24 93
Carbohydrates (as glucose), per cent	17 82	17 42	2 06	0 75
Relative viscosity	1 12	1 12	1 20	1 23
Specific rotation $(\alpha)_D$ 22°	47 8°	49 8°	11 0°	4 7°
Biuret *	±	+	—	—
Bial *	—	—	—	—
Dische *	—	—	—	—
Millon *	—	—	—	—
Glucosamine	+	+	+	+
Muramic acid	+	+	+	+
Glucose †	(+)	+	(+)	±
Glutamic acid	+	+	+	+
Serine	+	+	+	+
Glycine	+	+	+	+
Alanine	+	+	+	+
Lysine	+	+	+	+
Ribitol	—	—	+	+
Anhydroribitol		—	+	+
Glycerol	+	+	—	—

* Concentration of polysaccharide 5 mg/ml

† Brackets denote that the sugar found is assumed to be glucose

SUMMARY

Antigenic polysaccharide materials have been extracted from disrupted cells of four *Staph epidermidis* strains Further purification of the material has been obtained on ion exchange columns

Five amino acids, alanine, glycine, glutamic acid, lysine, and serine together with glucosamine and muramic acid have been found in each of the preparations Glucose seemed to be present in appreciable amounts in two preparations whereas the two other preparations contained only negligible amounts Glycerol has been found in two of the polysaccharides and ribitol in the other two, which indicates two types of teichoic acids

The one type, supposed to be a glycerol teichoic acid mucopeptide, was shown to have a specific rotation of 48–50° and a carbohydrate content of 17 18 per cent, whereas the corresponding values for the other, supposed to be a ribitol teichoic acid mucopeptide, were 5–11° and 2 per cent or less

The glycerol type shows definite similarity to the *Staph epidermidis* carbohydrate B of *Julianelle & Wieghard* (26), whereas the ribitol type seems to be structurally similar to polysaccharide A from *Staph aureus*, but may have different glycosidic linkages

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difference in the amount of glucose, as this sugar was the only one found on qualitative estimation It seems reasonable to assume that the weak reactions for glucose found in polysaccharides 3519 and 1622 are due to contaminants

The reducing capacities found are greater than those that can be calculated from the hexosamine and carbohydrate content Interaction of amino acids may render the reducing value too high On the other hand, the hexosamine content, determined as glucosamine, is likely to be too low as the muramic acid present, according to *Crumpton* (7), only produces about 27 per cent of the colour intensity of an equal weight of glucosamine at 530 mμ Furthermore, with the conditions of hydrolysis employed, there were still small amounts of unhydrolyzed, Elson-Morgan reacting material left as pointed out by paper chromatography

With milder conditions of hydrolysis, our amino sugars, estimated on paper chromatograms, probably would have appeared to be N-acetyl derivatives since most naturally occurring amino sugars contain N-acetyl groups

The amino acids alanine, glycine, glutamic acid, lysine, and serine, as demonstrated in each of our polysaccharides, are regularly found in the walls of staphylococci (34), and constitute, together with glucosamine and muramic acid, the mucopeptide of the wall

According to *Haukenes* (17, 20) polysaccharide A constitutes a part of the cell wall of *Staph aureus* and is composed of two structural components, a mucopeptide and a ribitol teichoic acid The present examinations indicate that the purified polysaccharide material isolated from *Staph epidermidis* strains 1254 and 1268 contains a glycerol teichoic acid-mucopeptide, whereas the polysaccharide from *Staph epidermidis* strains 1622 and 3519 contains a ribitol teichoic acid-mucopeptide

The analytical data from polysaccharides 1622 and 3519 are very similar to those found by *Haukenes* (16–20) in polysaccharide A The difference in optical rotation, a dextrorotation of +5 to 11° in polysaccharides 1622 and 3519 compared to a laevorotation of —11 5° in polysaccharide A (16), may, however, be of importance The small amounts of glucose found in polysaccharides 1622 and 3519 may influence the optical rotation There is also a possibility that the difference is due to the glycosidic linkages present, e g that polysaccharides 1622 and 3519 contain more α-glycosidic linkages than polysaccharide A In the subsequent paper on the antigenic properties of polysaccharide 1622 and 3519 it will be shown that this polysaccharide also has the α glycosidic linkages, which determine the serologic specificity of polysaccharide A (21)

The values obtained for optical rotation, carbohydrates, and fermentable sugars in our polysaccharides 1254 and 1268 are in good agreement with the values reported by *Wieghard & Julianelle* (37) for carbohydrate B

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IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDES FROM STAPHYLOCOCCUS EPIDERMIDIS

2. *Antigenic Properties*

By

NORVALD LOSNEGARD and PER OEDING

Received 4 i 63

The information on the antigenic properties of *Staphylococcus aureus* has recently been reviewed by *Oeding* (10). Little is, however, known of the antigenic properties of coagulase negative staphylococci.

Julianelle & Wieghard (6) reported that pathogenic and nonpathogenic staphylococci could be differentiated by means of specific carbohydrate precipitinogens which they called A and B respectively. *Thompson & Khorazo* (13) described additional types of nonpathogenic staphylococci, C, BC, and O. These authors and *Verwey* (14) found type A strains to be mannitol-, α-toxin-, and coagulase positive, whereas B, C, BC, and O strains were negative with these tests. Unfortunately these type strains seem to have been lost and a comparison with carbohydrates A and B has to be based mainly on chemical characterization (15). *Haukenes* (2) demonstrated that of 100 *Staph. aureus* strains 97 produced the polysaccharide A line on agar precipitation, whereas all the 62 *Staph. epidermidis* strains examined by the same technique, failed to give this line. Ten of the latter strains were also examined by a more sensitive technique. He was then able to demonstrate the polysaccharide A line in three of these strains.

In a previous paper (7) the chemical characteristics of two types of purified polysaccharide from strains of *Staph. epidermidis* have been described. One type was thought to be a glycerol teichoic acid mucopeptide and showed definite similarity to *Julianelle & Wieghard*'s (6) carbohydrate B, whereas the other, thought to be a ribitol teichoic acid mucopeptide, seemed to be structurally similar to polysaccharide A of *Staph. aureus*. In the present paper these polysaccharides and a number of other *Staph. epidermidis* strains have been examined serologically.

MATERIALS AND METHODS

Strains. One hundred and twenty five coagulase negative *Staph. epidermidis* strains from our routine material, including the strains 1254, 1268, 1622 and 3519 chemically characterized in (7), were examined. Four pathogenic *Staph. epidermidis*

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IMMUNOCHEMICAL STUDIES ON POLYSACCHARIDES FROM STAPHYLOCOCCUS EPIDERMIDIS

2 *Antigenic Properties*

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Julianelle & Wieghard (6) reported that pathogenic and nonpathogenic staphylococci could be differentiated by means of specific carbohydrate precipitinogens which they called A and B respectively *Thompson & Khorazo* (13) described additional types of nonpathogenic staphylococci C, BC, and O These authors and *Verwey* (14) found type A strains to be mannitol , α toxin- and coagulase positive, whereas B, C BC, and O strains were negative with these tests Unfortunately these type strains seem to have been lost and a comparison with carbohydrates A and B has to be based mainly on chemical characterization (15) *Haukenes* (2) demonstrated that of 100 *Staph aureus* strains 97 produced the polysaccharide A line on agar precipitation, whereas all the 62 *Staph epidermidis* strains examined by the same technique, failed to give this line Ten of the latter strains were also examined by a more sensitive technique He was then able to demonstrate the polysaccharide A line in three of these strains

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ca[illegible]
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MATERIALS AND METHODS

Strains One hundred and twenty five coagulase negative *Staph epidermidis* strains from our routine material including the strains 1254 1268 1622 and 3519 chemically characterized in (7) were examined Four pathogenic *Staph epidermidis*

strains were supplied by *Maclean Smith* Iowa City (12) The *Staph aureus* type strain 1503 (8) was used for comparison

Purified polysaccharide material was prepared from strains 1254 1268, 1622 3519, and 1503 (7)

Immune sera against the 5 strains above were produced by intravenous injections of formalin-killed bacteria by *Oeding's* (9) method

Absorption was carried out as described by *Haukenes* (2)

Precipitation using the ring test was performed by the method used by *Haukenes et al* (4)

Agar diffusion technique The agar medium and the plates were prepared as described in (5) The thickness of the agar gel was about 2 mm One central and 6 peripheral, circular basins of 12 mm diameter were cut, the distance between them being 10 mm The technique used for the examination of the purified polysaccharides has been described in (5)

A special technique which offered good opportunities for observing the characteristic polysaccharide lines was used for the examination of the routine strains All strains were examined against antisera 1254 3519, and 1503 using polysaccharides 1254 3519, and 1503 as references Alternate peripheral basins were filled with glucose broth and inoculated with the strains to be tested After incubation at 37° C for 24 hours the central basin was filled with antiserum Six hours later the polysaccharide, usually in a concentration of 0 1 mg/ml was put into the remaining peripheral basins

Though it produces the same polysaccharide lines antiserum 3519 was preferred to antiserum 1622 as the latter was a rather weak antiserum Antisera 1254 and 1268 possessed the same antibodies The former was selected as it appeared to be the stronger one, producing more distinct lines

RESULTS

Ring Test Precipitation

According to the strong cross precipitation (Table 1) there must be a close relationship between polysaccharides 1254 and 1268, and also between those of 1622, 3519, and 1503 This is confirmed by the results of agar gel precipitation (see below) Some cross-precipitation also takes place between polysaccharides of the two groups, the titres for polysaccharide 1254 against antiserum 1503 and polysaccharide 1622 against antiserum 1268 being rather high

TABLE 1

Ring Test Precipitation with Staphylococcal Polysaccharide Materials

Antiserum	1254	1268	1622	3519	1503
1254	1 4 000	1 3 000	1 100	1 100	1 100
1268	1 4 000	1 3 000	1 100(0)	1 100	1 100
1622	1 10	1 10	1 2 000	1 3 000	1 1 000
3519	1 100	1 100	1 1 000	1 3 000	1 2 000
1503	1 100(0)	1 100	1 2 000	1 3 000	1 6 000

The titre indicates the highest dilution of antigen (mg ml) giving precipitation Antiserum undiluted

According to the absorption experiments, polysaccharides 1622 and 3519 seem to have one rather strong antigen which is not shared by the other polysaccharides This is apparently the case also with polysaccharides 1254 and 1268 Polysaccharide A (1503), however, does

not seem to have any antigenic specificity which is not also found in the 1622 and 3519 polysaccharides As the sera used for these experiments are produced against the whole organisms, the relationship between the polysaccharide materials is very difficult to evaluate Specific sera have not been obtained as the polysaccharides were non antigenic in rabbits

Agar Gel Precipitation

The behaviour of the polysaccharide preparations on agar gel diffusion is shown in Fig 1–3 Each of the preparations produces more than one line against the homologous serum Only those lines which seem to be definitely established are illustrated in the figures In accordance with the precipitation reactions (Table 1), polysaccharides 1254 and 1268 behave identically on agar diffusion also, as both the observed lines give a reaction of identity Polysaccharide 1622 has four lines in common with polysaccharide 3519 against antiserum 3519, two of which are also produced by polysaccharide 1503 (Fig 3) The line designated I is weak and not always easy to demonstrate A strong antiserum is required as the antigenic part of the system migrates rapidly into the antibody basin Line II, which is also demonstrated against antiserum 1503 (Fig 2) is the polysaccharide A line Polysaccharide A is apparently responsible for the strong cross precipitation observed in the ring test between strain 1503 and strains 1622 and 3519 Lines III and IV (Fig 3) are regarded as the principal lines of strains 1622 and 3519

After absorption of sera 1622 and 3519 with polysaccharide 1268 and polysaccharide 1503 these sera still produced lines III and IV against polysaccharide 1622, whereas polysaccharide 1503 gave no line As polysaccharide 1268 had not been shown to give a polysaccharide A line (II) its ability to remove the corresponding antibody seemed

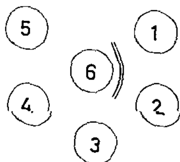


Fig 1

Schematic drawing of 1 ...
Polysaccharide
1 2 3 and 5 re...
Antiserum 1254

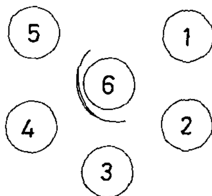


Fig 2

Schematic drawing of lines produced against antiserum 1503 on agar gel diffusion Polysaccharide from *Staph epidermidis* strains 1268 1254, 1622 and 3519 in wells 1, 2, 3, and 5 respectively Polysaccharide from *Staph aureus* strain 1503 in well 4 Antiserum 1503, undiluted, in well 6 All polysaccharides in 0 1 mg/ml concentration

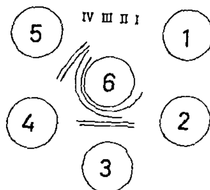


Fig 3

Schematic drawing of lines produced against antiserum 3519 on agar gel diffusion Polysaccharide from *Staph epidermidis* strains 1268 1254, 1622 and 3519 in wells 1, 2, 3 and 5 respectively Polysaccharide from *Staph aureus* strain 1503 in well 4 Antiserum 3519 *undiluted in well 6 All polysaccharides in 0 1 mg/ml concentration*

strange, although analogous results had been obtained on ring test precipitation The following experiment may also indicate that polysaccharides 1254 and 1268 have small amounts of polysaccharide A or that they have part of a structure in common with polysaccharide A Antisera 1622 and 3519 absorbed with excess polysaccharide 1268, produced a line which gave a probable reaction of identity with polysaccharide A against antiserum 1503 This should be compared with the findings of *Haukenes* (2) mentioned in the introduction It is also possible that line I *is present in* polysaccharides 1254 and 1268 These possibilities await further investigation

In Table 2 are listed the results of agar gel precipitation with *125* strains of *Staph epidermidis* selected from our routine material *All* strains were tested against reference systems 1254, 3519, and 1503 The strains were classified according to the lines showing reactions of

identity with the reference polysaccharide lines (Fig 1–3), although line I was excluded Thus, the designation group A means that a strain has the polysaccharide A line exclusively, and group B that a strain has the double line found in polysaccharides 1254 and 1268 The double line characteristic of polysaccharides 1622 and 3519 has been designated C, and because these polysaccharides also have an A line, the group designation is AC

TABLE 2

Examination of Routine Strains of Staph epidermidis by Agar Gel Diffusion

No of strains	Origin	Group according to polysaccharide lines					
		A	B	AB	AC	ABC	0
36	Nose	2	27	0	6	1	0
6	Throat	1	3	0	0	1	1
14	Pus	0	7	0	4	0	3
9	Blood	0	5	0	1	1	2
9	Others	0	5	0	0	0	4
Sum 74		3	47	0	11	3	10
30*	Urine	1	13	0	8	2	6
21†	Urine	0	3	0	16	1	1
Sum 51		1	16	0	24	3	7
Total 125		*4*	*63*	*0*	*35*	*6*	*17*

Typing was based on a reaction of identity with the reference polysaccharide
Group O strains produced no line
* Strains with no or a dubious relationship to infection
† Strains with possible relationship to infection

The strains were selected with the intention of comparing the precipitinogens present in strains which had almost certainly *no pathogenic significance*, with strains which were considered to be the causative agents of the infections from which they were isolated If a sample of pus or urine showed Gram positive cocci in heaps and leucocytes microscopically and gave a pure culture of *Staph epidermidis, it was considered* possible that the strain was responsible for the infection

Table 2 shows a certain correlation between the polysaccharide group and the origin of the material The most striking difference is noticed between the nose strains and the strains considered to be responsible for urinary infections *Seventy-five per cent of the nose strains belong* to group B whereas 76 per cent of the urinary strains, *group 2*, are AC strains The 4 strains registered under group A may have small amounts of C antigen and thus belong to group AC

Four strains of *Staph epidermidis* which have been reported by *Smith et al* to have caused septicemia (12), were also examined All these strains were of polysaccharide group B

DISCUSSION

A previous article (7) dealt with the chemical characterization of the polysaccharides. Polysaccharides 1254 and 1268 were found to belong to one group, whereas polysaccharides 1622 and 3519 belonged to another. This is supported by their antigenic properties.

Polysaccharides 1622 and 3519 seem structurally to be very similar to polysaccharide A, the only significant difference being dextrorotation in the former and laevorotation in the latter (7). The present investigations demonstrate at least two serological specificities of polysaccharides 1622 and 3519, one being the same as that of polysaccharide A. The other specificity may also be found in the ribitol teichoic acid structure, and as β-glycosidic linkages are responsible for the serological specificity of *Staph. aureus* teichoic acid (3), the possibility must be considered that α-linkages are responsible for the other specificity. The serological activity may also be ascribable to the mucopeptide moiety.

The polysaccharide of *Staph. epidermidis* strains 1254 and 1268 is probably a glucose-glucosamine glycerol teichoic acid-mucopeptide. A wall teichoic acid of similar composition was isolated by *Ghuysen* (1) from a strain of *B. megaterium*. The strong specific serologic reactivity of our polysaccharide may be ascribable to the glucose linkage, whereas the weak cross-reaction with polysaccharide A and polysaccharides 1622 and 3519, can be explained by the presence of β-glucosamine linkages.

The significance of the line designated I (Fig. 3) is at the moment rather obscure, but the antigen involved seems to be widely distributed among the strains examined. The question arises whether this antigen may play a part in some of the cross reactions observed between strains from different groups.

Due to the chemical composition (7) and antigenic properties of their polysaccharides, the strains 1254 and 1268 are regarded as group B strains, i.e. type B strains of *Julianelle & Wieghard* (6). It may be reasonable to use the designation AC for the serologic reactivities demonstrated in polysaccharides 1622 and 3519, until the chemical nature of the active groups has been definitely determined.

The examination of 125 routine strains of *Staph. epidermidis* revealed a certain correlation between polysaccharide group and origin of the strains. Although there may be other reasons for this distribution, the findings might indicate that certain types of *Staph. epidermidis* are associated with the place of growth. It is also possible that strains capable of causing infections have different polysaccharide antigens than purely saprophytic strains. It has been shown in the present investigation that one group of *Staph. epidermidis* strains possess polysaccharide A, which is otherwise characteristic of *Staph. aureus*. However, it seems that in these strains polysaccharide A is a minor antigen, and

always (or nearly always) associated with another, major antigen (C) *Staph aureus* strains always seem to have polysaccharide A. One might therefore think that polysaccharide A is associated with the ability to cause infection. If so, pathogenic strains of *Staph epidermidis* should have polysaccharide A as a minor antigen. The results shown in Table 2 seem to support this theory as 76 per cent of the group of urinary strains with possible relation to infection are of polysaccharide group AC. In contrast the majority of the nose strains, which should be expected to be saprophytic, belong to group B. However, 4 strains reported by *Smith et al* (12) to have been responsible for cases of septicemia, were shown by us to be polysaccharide B strains. Recently *Pereira* (11) reported that strains of *Staph epidermidis* responsible for urinary infections, all had a certain agglutinogen, but the chemical nature of this is unknown.

It is accepted that *Staph epidermidis* may be able under certain circumstances to cause infections in man. Therefore, it would be of interest to extend our very poor knowledge of the antigens of these organisms.

SUMMARY

Purified polysaccharide preparations from four *Staph epidermidis* strains, examined by ring test and agar gel precipitation were found to comprise two serological groups, B and AC. This is in accordance with the chemical data presented in a previous article. There was strong cross precipitation between polysaccharide A of *Staph aureus* (group A) and the AC polysaccharide, due to the presence of polysaccharide A in *Staph epidermidis* group AC strains. The purified *Staph epidermidis* polysaccharides thus have more than one serological specificity.

The AC polysaccharide is thought to contain glucosamine ribitol teichoic acid with β glykosidic linkages that react serologically with polysaccharide A and another grouping determining the specificity of the substance. The B polysaccharide probably contains a glycerol teichoic acid with glucose linkages which may determine the strong specific reaction and β-glucosamine linkages which may give cross reactions.

A number of routine strains of *Staph epidermidis* were examined for the presence of the group polysaccharide antigens A, B, and AC. In this material the majority of the presumed saprophytic nose strains belonged to group B, whereas *group AC was the most common among* presumably pathogenic urinary strains.

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DISCUSSION

A previous article (7) dealt with the chemical characterization of the polysaccharides Polysaccharides 1254 and 1268 were found to belong to one group, whereas polysaccharides 1622 and 3519 belonged to another This is supported by their antigenic properties

Polysaccharides 1622 and 3519 seem structurally to be very similar to polysaccharide A, the only significant difference being dextrorotation in the former and laevorotation in the latter (7) The present investigations demonstrate at least two serological specificities of polysaccharides 1622 and 3519, one being the same as that of polysaccharide A The other specificity may also be found in the ribitol teichoic acid structure, and as β-glycosidic linkages are responsible for the serological specificity of *Staph aureus* teichoic acid (3), the possibility must be considered that α-linkages are responsible for the other specificity The serological activity may also be ascribable to the mucopeptide moiety

The polysaccharide of *Staph epidermidis* strains 1254 and 1268 is probably a glucose-glucosamine glycerol teichoic acid-mucopeptide A wall teichoic acid of similar composition was isolated by *Ghuysen* (1) from a strain of *B megaterium* The strong specific serologic reactivity of our polysaccharide may be ascribable to the glucose linkage, whereas the weak cross-reaction with polysaccharide A and polysaccharides 1622 and 3519, can be explained by the presence of β glucosamine linkages

The significance of the line designated I (Fig 3) is at the moment rather obscure, but the antigen involved seems to be widely distributed among the strains examined The question arises whether this antigen may play a part in some of the cross reactions observed between strains from different groups

Due to the chemical composition (7) and antigenic properties of their polysaccharides, the strains 1254 and 1268 are regarded as group B strains, *i e* type B strains of *Julianelle & Wieghard* (6) It may be reasonable to use the designation AC for the serologic reactivities demonstrated in polysaccharides 1622 and 3519, until the chemical nature of the active groups has been definitely determined

The examination of 125 routine strains of *Staph epidermidis* revealed a certain correlation between polysaccharide group and origin of the strains Although there may be other reasons for this distribution, the findings might indicate that certain types of *Staph epidermidis* are associated with the place of growth It is also possible that strains capable of causing infections have different polysaccharide antigens than purely saprophytic strains It has been shown in the present investigation that one group of *Staph epidermidis* strains possess polysaccharide A, which is otherwise characteristic of *Staph aureus* However, it seems that in these strains polysaccharide A is a minor antigen, and

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TUBERCULIN PRODUCTION

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In preliminary (unpublished) experiments with heat-killed cultures of *Mycobacterium tuberculosis*, larger amounts of tuberculoprotein were extracted with weak sodium hydroxide than with saline or phosphate buffer Extraction was slow at room temperature but fairly quick at higher temperatures It was also found that the biological activity of tuberculin could be destroyed by prolonged treatment with sodium hydroxide Therefore, in the present study extraction was carried out with weak sodium hydroxide (0 05 N)

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The four factors referred to in this plan are Strain age of culture and fraction, all at two levels and medium at three levels

The assay included 96 guinea pigs and each animal received eight tuberculin injections six test tuberculins selected according to the plan and the two standard dilutions Thus every test tuberculin was used for 24 injections

Tuberculin injection The injections were given into eight sites located in a zigzag row on the two flanks All injections were given intradermally by the same person and the volume injected was 0 2 ml

Reading The reactions were read after 24 and 48 hours The size of the erythema (in mm) was measured with a ruler and the average of two diameters at right

[illegible]

formula

$$\log D_{obs} = \frac{\overline{TR}_T - \overline{TR}_{ST}}{\overline{b}_{ST}} + 1.5$$

where $\overline{TR}_T$ = average tuberculin reaction for the test tuberculin

$\overline{TR}_{ST}$ = average tuberculin reaction for the two standard doses and

b_{ST} = average slope[1] of the tuberculin dose-response curve = difference

[illegible] groups For each [illegible] the three groups [illegible] for the individual group and the same b_{ST} for all groups Estimates of the standard error (SE) of $\log D_{obs}$ are obtained from these sets of three values

The activity D (in units per ml) of the culture filtrates is calculated from $D = 1000 \times \overline{D_{obs}}$ and the activity of the extracts from $D = 1000 \times \overline{D_{obs}} \times \frac{1}{10}$ (dilution factor) where $\overline{D_{obs}}$ is the geometric mean of the three determinations of D_{obs}. The 95 per cent confidence limits for the activity are $D \times 10^{\pm 2 SE}$

RESULTS

Protein analyses Table 1 shows the dry weight of organism (in mg per flask) for the cultures and the protein yields (in mg) for the culture filtrates and the extracts, calculated per ml of culture filtrate

Taking into consideration that the protein analyses for the glycerol broth culture filtrates are not comparable with the other protein analyses since they include the meat protein of the broth, (*Magnusson, Kim & Bentzon* 1963), it will be seen from the table that more protein was extracted from the cells than was present in the culture filtrates, except for strain F9606 cultured on Lind bII medium

More protein was extracted from 8 weeks-old cultures [illegible]

[illegible] and Lind bII medium

[1] The average slope was used because there were only random variations in the slopes for the individual animals

MATERIALS AND METHODS

Cultures Two virulent strains of *Mycobacterium tuberculosis* T3487 and F9656 were cultured at 38° C for four and eight weeks in small flasks containing 180 ml of glycerol broth Sauton or Lind bII medium (*Lind* 1948) The cultures were sterilized by heating in streaming steam for one hour The bacteria were filtered off on paper and dried before extraction The cultures were included in a previously published study (*Magnusson Kim & Bent on* 1963) where further details are given regarding the cultures

Extraction 200 mg of dried cells were suspended in 7 ml of 0 05 N sodium hydroxide and boiled for 30 minutes cooled and centrifuged 5 ml of the supernatant was drawn off by pipette while another 5 ml of 0 05 N sodium hydroxide was added to the bacterial suspension and the extraction repeated To prevent destruction of the tuberculin activity part of each extract was immediately diluted with buffer and sterilized by autoclaving before being used in the biological assay Another part of the extract was precipitated with trichloroacetic acid for protein determination In all five extractions of each bacterial sample were carried out After all the extractions were finished the extracts originating from the same bacterial specimen were mixed and used for the biological assay or protein determination

Protein determination Determination of protein was made by the biuret reaction as described previously (*Magnusson Kim & Bent on* 1963) For the culture filtrates the protein yield is calculated as follows protein yield (in mg per ml) $= \frac{E}{0\,110} \times \frac{1}{10}$ (E = extinction observed 0 110 = extinction for 1 mg/ml protein and $\frac{1}{10}$ is a dilution factor)

For the extracts the protein yield (per ml of culture filtrate) is calculated from protein yield (in mg per ml) $= \frac{E}{0\,110} \times 5 \times \frac{W}{200} \times \frac{1}{150}$ (5 is the dilution factor for extracts and $\frac{1}{150} \times \frac{W}{200}$ is due to the extraction being made on 200 mg of a total of W mg cells obtained from 150 ml culture filtrate 150 ml is an estimate of the average amount of culture filtrate obtained from 180 ml medium)

Biological assay The biological assay included a comparison of the activity of the 12 extracts and the corresponding culture filtrates by intradermal testing on BCG vaccinated guinea pigs These 24 preparations were compared with tuberculin dilutions containing 10 tuberculin units (TU) and 100 TU per 0 2 ml Purified tuberculin RT 22 from Statens Seruminstitut was used as standard tuberculin 1 mg of RT 22 was assumed to be equal to 75 000 TU

Tuberculin dilutions The intention was to use such concentrations of the test tuberculins as would give skin reactions of a size between the reactions to the two standard doses Preliminary assays revealed that appropriate reactions could be obtained when the culture filtrates were diluted 1 200 and the extracts 1 $\frac{24000}{W}$

Fresh dilutions were prepared on each testing day Sterile phosphate buffered saline was used as diluent (One litre sterile buffer contains 1 45 g KH_2PO_4 + 7 60 g $Na_2HPO_4\,2H_2O$ + 4 8 g NaCl)

Experimental animals White BCG vaccinated guinea pigs weighing 530 650 g were used Each animal was vaccinated intracutaneously in four places on the abdomen with 0 1 ml of Danish BCG vaccine[1] ten times stronger than the standard vaccine Thus each animal received 3 mg of (semi dried weight) BCG The vaccine batches were Nos 1141 1143 The animals were used five to six weeks after vaccination

Experimental design It is known that the tuberculin sensitivity in a group of sensitized animals varies from one animal to another and even within an animal the sensitivity varies according to the test site Therefore in order to ensure accurate comparison between all the 24 test preparations with a limited number of animals a special experimental design was used The design (not shown) is a modification of that described in *Cochran & Cox* (1955) Experimental Designs p 216 Plan 6 10

1 The authors are grateful to Dr *K Tolderlund* and Miss *K Bunch Christensen* BCG Department Statens Seruminstitut for preparing and supplying the vaccine

TABLE 2

Mean Size of Intradermal Reactions Read after 24 Hours (Erythema in mm) of BCG Vaccinated Guinea Pigs and Tuberculin Yields (in Tuberculin Units per ml) of Culture Filtrates and Extracts of two Strains of Mycobacterium tuberculosis Cultured on three Different Media

Culture			Culture filtrate		Extract	
Strain	Medium	Age of culture (weeks)	Mean reaction* 24 hours (mm)	Tuberculin yield † ° D (TU)	Mean reaction § 24 hours (mm)	Tuberculin yield † ° D (TU)
T3487	Glycerol broth	4	16 6	25 000	15 1	1,300
		8	19 4	80 000	18 1	4,500
	Sauton	4	18 8	60 000	18 2	4 500
		8	19 4	80 000	20 3	11 000
	Lind bII	4	18 1	45 000	17 4	3 500
		8	19 8	90 000	20 2	11 000
E3656	Glycerol broth	4	20 0	100 000	17 2	3 000
		8	20 2	110 000	18 1	4 500
	Sauton	4	17 7	40 000	21 1	10 500
		8	18 9	65 000	20 5	12 500
	Lind bII	4	20 1	105 000	19 2	7 000
		8	21 6	195 000	20 7	13 500

Mean reaction (96 reactions) to standard solution containing 100 TU/0 2 ml 20 0 mm
Mean reaction (96 reactions) to standard solution containing 10 TU/0 2 ml 14 5 mm

* Each figure is the mean of 24 reactions Dilution rate 1 200
§ Each figure is the mean of 24 reactions Dilution rate 1 $\frac{24000}{W}$ see text
† The 95 per cent limits of D are 0 70 × D and 1 43 × D
° Calculated per ml of culture filtrate

graphically in Fig 1 As regards the extracts (Fig 1, upper part), the points are located on a line with slope 1 This means that the biological activity is proportional to the amount of extracted protein, or in other words that all the extracts show the same biological activity per mg protein

However as regards the culture filtrates (Fig 1, lower part) the points do not fit a line with slope 1 Generally, when much protein is formed this is less active (per mg) than when the amounts are smaller

Relationship between total amount of tuberculin and growth of the bacteria In order to test the relationship between the total amount of tuberculin obtained from a culture and the weight of the culture, the logarithm of the total tuberculin activity ($T_{cf} + T_{ex}$) for each culture has been calculated In Fig 2 these values have been plotted against the weight of the cultures

Even though there is some relationship between the total amount of tuberculin formed and the weight of the culture, the deviations of the points from a straight line are larger than might be expected with the actual experimental error

TABLE 1

Dry Weight of Organism (in mg) and Protein Yields (in mg per ml) in Culture Filtrates and Extracts of two Strains of Mycobacterium tuberculosis Cultured on three Different Media

Culture			Dry weight of organism per flask* (mg)	Protein yield (mg)	
Strain	Medium	Age of culture (weeks)		Culture filtrate	Extract †
T3487	Glycerol broth	4	330	0.33§	0.13
		8	820	0.50§	0.43
	Sauton	4	1350	0.16	0.43
		8	1350	0.16	1.25
	Lind bII	4	1000	0.16	0.35
		8	2100	0.36	1.34
F9656	Glycerol broth	4	570	0.49§	0.31
		8	820	0.09§	0.62
	Sauton	4	1500	0.07	0.67
		8	1320	0.19	0.92
	Lind bII	4	1900	0.73	0.60
		8	2000	1.43	1.45

* 180 ml medium
§ Unreliable, includes meat protein from glycerol broth
† Calculated per ml of culture filtrate, see text

Biological activity The amount of tuberculin obtained by extraction of the cells is smaller than that present in the culture filtrate (Table 2) On an average, extraction yields only about 10 per cent of the amount of tuberculin present in the culture filtrates The percentage depends on the age of culture, strain and kind of medium, varying from about 3 per cent (4 weeks' culture of E9656 on glycerol broth) up to 27 per cent (4 weeks' culture of E9656 on Sauton medium)

More tuberculin was obtained from 8 weeks-old than from 4-weeks-old cultures Lind bII medium gave systematically higher yields of tuberculin than glycerol broth, and there were no systematic differences in the yield between the two strains

The yields calculated on the basis of observations after 48 hours (Table 3) were systematically lower than after 24 hours (Table 2) However, the relationship between the test tuberculins was much the same after 48 hours as after 24 hours The use of one set of average values ($\overline{b}_{ST}$ and $\overline{IR}_{ST}$) for all the 24 hour comparisons and another set for all the 48-hour comparisons may account for the systematic trend This difference has not been observed in later studies

Relationship between protein content and biological activity The logarithms of the amount (in mg) of protein per g of dried weight of organism or per ml culture filtrate, and the tuberculin activity (in TU) per g of dried weight of organism or per ml culture filtrate, have been calculated for the 24 tuberculin preparations The results are shown

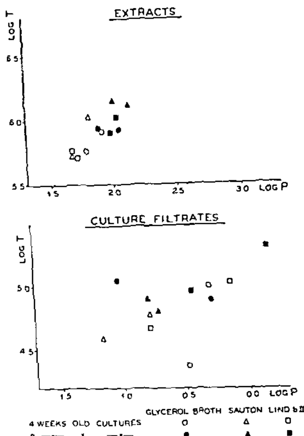


Fig 1

Tuberculin activity in relation to protein content of extracts and culture filtrates of *Mycobacterium tuberculosis* cultured on three different media

Abscissa Logarithm of the protein content in mg per g of dried weight of organism or per ml culture filtrate

Ordinate Logarithm of the tuberculin activity in TU per g of dried weight of organism or per ml culture filtrate

was much bigger from Lind bII than from Sauton medium However, the biological activity of the preparation per mg substance was correspondingly lower, and there was little difference in the total tuberculin activity obtained on the two media In the present experiment, a higher yield of tuberculin was obtained on Lind bII medium than on glycerol broth Usually the tuberculin yield was also higher on Lind bII medium than on Sauton The average yield on Sauton was about 60 per cent of that on Lind bII The yields of tuberculin differ less than the yields of tuberculoprotein on the two media

According to *Green* (1946), the protein content is a reliable measure of the biological activity of tuberculin preparations However, *Seibert*,

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T3487	Glycerol broth	4	13.1	15 000	11.0	800
		8	16.7	60 000	15.4	7 500
	Sauton	4	16.2	50 000	15.5	4 000
		8	16.4	50 000	17.6	8 000
	Lind bII	4	15.5	40 000	14.4	2 500
		8	17.6	80 000	18.0	9 000
T9656	Glycerol broth	4	17.1	65 000	14.9	3 000
		8	17.1	65 000	16.3	5 000
	Sauton	4	15.2	35 000	17.6	8 000
		8	16.3	50 000	17.6	8 000
	Lind bII	4	17.3	70 000	16.3	5 000
		8	19.3	140 000	18.2	9 500

Mean reaction (96 reactions) to standard solution containing 100 TU/0.2 ml 17.3 mm
Mean reaction (96 reactions) to standard solution containing 10 TU/0.2 ml 10.7 mm

* Each figure is the mean of 24 reactions. Dilution rate 1:200

§ Each figure is the mean of 24 reactions. Dilution rate 1: $\frac{24000}{W}$ — see text

† The 95 per cent limits of D are 0.67 × D and 1.49 × D

° Calculated per ml of culture filtrate

DISCUSSION

Arena (1938) made direct comparison between the amount of tuberculin extracted from the cells and the amount present in the culture filtrate and found a larger amount in the extracts. In the present experiment, fairly large amounts of tuberculoproteins have been extracted from tubercle bacilli. The biological activity of the extracts, calculated per mg protein, is constant but so small that the total activity of the extracts is smaller than the activity of the corresponding culture filtrates. Consequently, it is of no advantage to prepare tuberculin by extraction of the cells by means of the present extraction method. A modified procedure for extraction is to alkalize the medium after growth of the bacilli and extract the tuberculin from the cells by subsequent heat sterilization of the cultures. In this way, the culture filtrate will include the extracted proteins. This procedure has been used in a subsequent study in this series.

Lind (1948) found that the yield (by weight) of purified tuberculin

and the tuberculin activity of the extracts were compared with the protein content and the activity of the corresponding culture filtrates

Large amounts of protein were extracted from the cells, but the biological activity of the extracts was low The average activity of the extracts was ten times smaller than the activity of the culture filtrates

On the whole, more tuberculin was obtained on Land bH medium than on glycerol broth and Sauton medium Under varying experimental conditions, the amount of tuberculin obtained from 8-weeks-old cultures was larger than from 4-weeks-old cultures The relationship of the amount of tuberculin obtained with the two strains was dependent on the medium used

As regards the extracts, all the protein had the same biological activity, as regards the culture filtrates, the activity was higher in one part of the protein than in another

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Fig 2

Total tuberculin activity of culture filtrates and extracts in relation to bacterial crop of *Mycobacterium tuberculosis* cultured on three different media

Abscissa Logarithm of the dried weight of organism (in mg) per flask
Ordinate Logarithm of the total tuberculin activity ($T_{cf} + T_{ex}$) in tuberculin units per ml of culture filtrate

Figuero & Dufour (1955) have isolated tuberculoproteins with different biological activity per mg of protein, partly from unheated culture filtrates, and partly by extraction of tubercle bacilli In the present experiment, variations in the protein content of culture filtrates and extracts were obtained by using three different media, two strains and cultures of different ages The results indicate that some relation exists between protein content and tuberculin activity However, this is no simple relationship, since not all the protein has the same biological activity For instance, as mentioned above, the protein of Lind bII medium is not as active as that of Sauton medium, and the average activity of the extracts is only 5–10 per cent of that of the culture filtrates when calculated on the basis of the same amount of protein Thus, the present experiment shows that protein content cannot be used as a measure of the tuberculin activity under varying experimental conditions

SUMMARY

Two strains of *Mycobacterium tuberculosis* were cultured for four and eight weeks on glycerol broth, Sauton and Lind bII medium After heat sterilization of the cultures, the dried bacterial cells were extracted with 0 05 N sodium hydroxide The amount of protein extracted

FROM VIRUS DEPARTMENT A, NATIONAL INSTITUTE OF PUBLIC HEALTH, OSLO NORWAY

POLYOMA VIRUS

I Cell—Virus Interaction at Low Temperature

By

K Helgeland, O Lahelle and J Jonsen

Received 16 1 63

The rate of adsorption of SE polyoma to primary mouse embryo cells at 37° C was determined by *Winocour & Sachs* (1) Using a plaque assay they found a maximum adsorption after 3 hours *Crawford* (2), applying the same technique on the Toronto strain of polyoma virus, found no difference in the amount of virus adsorbed at 4°, 22°, and 37° C

Deichman (3) investigated the dynamics of SE polyoma virus multiplication in mouse embryo cells with cell virus suspensions which had been incubated for 18 hours at 4° C to ensure maximum virus adsorption No details are given to substantiate the validity of this procedure The present report deals with experiments intended to increase the yield of SE polyoma virus when grown in primary mouse embryo cells The effect of the temperature and of the incubation time for the initial cell virus interaction has been investigated in relation to the subsequent production of virus haemagglutinins by incubation of the infected cells at 37° C

MATERIALS AND METHODS

Virus A subline[1] of SE polyoma virus was used for all experiments The virus has undergone *several passages* in monolayer cultures of primary mouse embryo cells The source of virus was infectious tissue culture fluid clarified by low speed centrifugation

Cell cultures Primary mouse embryo cells were prepared by trypsinization of minced m… The cells were grown in 2[illegible]0 ml … ml of medium or in 600 ml R[illegible] … medium The cells were grown … [illegible] days at 37° C a confluent … as changed to Eagle Hanks … [illegible]

… add 20 ml to the Roux bottles)

When the cell virus *interaction* had taken place for a desired time the virus

[1] Originally obtained from Professor *Herbert Morgan* University of Rochester, and designated SE polyoma virus U.S.A.

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TABLE 2

Effect of Initial Cell Virus Interaction Temperature on Early Productions of Haemagglutinins

Interaction temperature	Extracellular HA on day				Intracellular HA on day	
	HA titre			Total HA units*	HA titre	Total HA units
	0	1	2	2	2	2
4°	0§	0	64	4800	256	2560
					128	1280
22°	0	0	64	4800	64	640
			32	2400	32	320
37°	0	0	8	600	0	0
			0	0		

* (HA units per ml) × volume § Less than 8

Each prescription bottle was inoculated with 7.5 ml of a virus suspension with a HA titre 1/256. The inoculum was replaced with 15 ml medium for virus growth. Intracellular HA was estimated after freezing and thawing the cells in 2 ml PBS.

intracellular haemagglutinins was found after 10 days in the 4° and 22° C cultures. The yield after incubation at 37° C is lower than the corresponding yields obtained after incubation at 4° and 22° C. This is the case for both extracellular haemagglutinins after various incubation times and for the intracellular haemagglutinins determined after 10 days.

A second experiment was made to investigate whether the early detectable difference in the amount of extracellular haemagglutinins in the 4°, 22°, and 37° C cultures was due to a different rate of release of virus from the cells. Table 2 shows the results of haemagglutinin titrations at the time of the first appearance of extracellular haemagglutinins. There is a difference in the amount of both extracellular and intracellular virus, with a maximum yield in the cultures where interaction took place at 4° C.

Effect of Cell-Virus Interaction Time on Virus Yield

The relationship between time and virus yield was investigated. The experiments were made with cells grown in prescription bottles, and the interaction time was varied from 1 to 300 minutes at 4° C and from 1 to 180 minutes at 22° C. Table 3 shows the results when the cell-virus interaction took place at 4° C. Higher titres both of extracellular and intracellular haemagglutinins are found when the time is increased from 1 to 20 minutes. Prolonged interaction time had no significant effect on the virus yield. The appearance of extracellular haemagglutinins at zero time in cultures with an initial interaction time of 180 and 300 minutes indicates, however, that the amount of virus attached to the cells may increase beyond 20 minutes.

Table 4 shows the results from a similar experiment carried out at 22° C. A time interval from 40 to 180 minutes gave the same amount of

suspension was removed and the cell layer and the adjoining glass surfaces were carefully washed two times with Eagle Hanks' *medium with 2 per cent horse serum* The volume of each washing was 7 5 ml and 25 ml respectively for the prescription bottles and the Roux bottles

Eagle Hanks medium with 2 per cent horse serum was then added and the cultures were incubated at 37° C

Estimation of virus Extracellular and intracellular haemagglutinins were estimated The extracellular *haemagglutinins* were determined in the medium after heating at 56° C for 30 minutes

To release intracellular *haemagglutinins* the monolayer was trypsinized the cells spun down at low speed centrifugation and the sediment suspended in phosphate buffered saline of pH 7 2 (*PBS*) The suspension was frozen and thawed four times and then clarified by low speed centrifugation The supernatant was heated at 56° C for 30 minutes *prior to titration*

Haemagglutinations were made by adding 0 2 ml of a 0 4 per cent suspension of guinea pig erythrocytes to 0 2 ml of serial two fold dilutions of a virus suspension PBS was used as diluent The erythrocytes were suspended in PBS containing 3 per *cent of a 30 per cent solution* of bovine serum albumin The haemagglutination was read after incubation at 4° C overnight

RESULTS

Effect of Cell-Virus Interaction Temperature on Virus Yield

The effect of the initial cell-virus interaction *temperature* was studied by infecting primary cell cultures with virus suspensions at 4°, 22°, and 37° C After 3 hours interaction at the respective temperatures the virus suspension was substituted with medium and the incubation continued at 37° C Aliquots of the medium for titrations of the zero time extracellular haemagglutinins were taken after 30 minutes to allow *any elution to occur before sampling Table 1* shows the effect of temperature variations on the virus production The yield of extracellular virus in cultures where the interaction took place at 4° C is identical to that obtained at 22° C when compared after incubation for 2, 8, and 10 days Slightly higher titres were observed after 4, 5, and 6 days in the 4° C cultures No significant difference in the amount of

TABLE 1

Effect of Initial Cell Virus Interaction Temperatures on Production of Haemagglutinins (HA)

Interaction temperature	Extracellular HA on day								Intracellular HA on day	
	HA titre							Total HA units*	HA titre	Total HA units
	0	2	4	5	6	8	10	10	10	10
4°	2	64	128	128	256	256	256	64000	4096	61440
					128				2048	30720
22°	0§	64	64	64	128	256	256	64000	4096	61440
37°	0	16	32	16	64	128	128	32000	1024	15360
		32								

* (HA units per ml) × volume § Less than 2

Each Roux bottle was inoculated with 20 ml of a virus suspension with a HA titre 1/128 The inoculum was *replaced with 50 ml medium for virus growth* Intracellular HA was estimated after freezing and thawing the cells in 3 ml PBS

time used for the initial cell-virus interaction When titrations were made on infected cultures which had been incubated for 1–10 days, a constantly lower titer was obtained when the interaction took place at 37° C compared with that obtained at 4° and 22° C The effect was not due to a decreased release of haemagglutinins as lower titres were obtained by estimation of both extracellular and intracellular haemagglutinins A difference in haemagglutinin titres was less pronounced by comparison of cultures kept at 4° and 22° C Significant higher titres were however found as an early event in the cultures where the initial cell virus interaction took place at 4° C This was especially the case for the intracellular haemagglutinins The experiments therefore indicate that a better yield of early virus is obtained when the temperature for the initial interaction of the virus with the cell is lowered from 37° to 4° C

The total amount of virus attached to cells in these experiments was too small to be detected by hamagglutinin titrations of the inoculum *before* and after attachment had taken place The experiment presented in Table 3 indicated, however, that some release of haemagglutinins occurred at 37° C from cells which had been kept at 4° C while no such release could be demonstrated with cells kept at 22° or at 37° C The release could be explained by two assumptions

a) a greater amount of virus being adsorbed at the lower temperature followed by

b) release of part of the adsorbed virus by elevating the temperature due to thermolability of the cell-virus binding

The increased virus yield by interaction at 4° C would then indicate that attachment with subsequent infection of the cells prevails above release compared with the effects of interaction which takes place at higher temperatures A more optimal infection of the cells could possibly be achieved by further studies on the effect of virus yield of variations in the rate of temperature change from 4° to 37° C

At low temperatures the Brownian movements of suspended virus particles are decreased due to the higher viscosity of the suspending medium (4) The number of virus particles colliding with the cells per unit time is therefore reduced An increased stability of the cell-virus bond obviously counteracts and exceeds the effect of viscosity change and is therefore probably responsible for the increased virus yield when the cell-virus interaction takes place at low temperature

As shown by Table 3 and 4, an attachment time of only 20 minutes at 4° C or 40 minutes at 22° C, gave maximal yield of virus haemagglutinins The results indicate, however, that there may be a time dependent attachment beyond 20 and 40 minutes The experiments by *Winocour & Sachs* (1) showed that an attachment time of 3 hours at 37° C was required to obtain a maximum number of plaques

haemagglutinins. A slightly decreased yield was obtained after interaction for only 1 to 20 minutes. The extracellular haemagglutinins found on the second day in cultures with an initial interaction time of 60 and 180 minutes *may indicate* that such prolonged interaction has some effect on the virus production.

TABLE 3

Effect of Initial Cell-Virus Interaction Time at 4° C on Early Production of Haemagglutinins

Interaction time (min.)	Extracellular HA on day				Intracellular HA on day	
	HA titre			Total HA units*	HA titre	Total HA units
	0	1	2	2	2	2
1	0§	0	64	4800	64	640
20	0	0	256	19200	256	2560
40	0	0	256	19200	512	5120
					256	2560
60	0	0	256	19200	512	5120
					256	2560
180	8	0	256	19200	512	5120
300	8	0	512	38400	512	5120
			256	19200		

* (HA units per ml) × volume § Less than 8

Each prescription bottle was inoculated with 7 5 ml of a virus suspension with a HA titre 1/256 The inoculum was replaced with 15 ml medium for virus growth Intracellular HA was estimated after freezing and thawing the cells in 2 ml PBS

TABLE 4

Effect of Initial Cell Virus Interaction Time at 22° C on Early Production of Haemagglutinins

Interaction time (min.)	Extracellular HA on day					Intracellular HA on day	
	HA titre				Total HA units*	HA titre	Total HA units
	0	1	2	3	3	3	3
1	0§	0	0	32	2400	16	160
				16	1200		
20	0	0	0	64	4800	32	320
				32	2400	16	160
40	0	0	0	64	4800	64	640
60	0	0	8	64	4900	64	640
						32	320
180	0	0	8	64	4800	128	1280
						64	640

* (*HA units per ml*) × volume § Less than 8

Each prescription bottle was inoculated with 7 5 ml of a virus suspension with a HA titre 1/256 The inoculum was replaced with 15 ml medium for virus growth Intracellular HA was estimated after freezing and thawing the cells in 2 ml PBS

DISCUSSION

The investigations show that the appearance of haemagglutinins produced at 37° C by monolayers of primary mouse embryo cells infected with SE polyoma virus depends upon the temperature and the

time used for the initial cell-virus interaction When titrations were made on infected cultures which had been incubated for 1–10 days, a constantly lower titer was obtained when the interaction took place at 37° C compared with that obtained at 4° and 22° C The effect was not due to a decreased release of haemagglutinins as lower titres were obtained by estimation of both extracellular and intracellular haemagglutinins A difference in haemagglutinin titres was less pronounced by comparison of cultures kept at 4° and 22° C Significant higher titres were however found as an early event in the cultures where the initial cell virus interaction took place at 4° C This was especially the case for the intracellular haemagglutinins The experiments therefore indicate that a better yield of early virus is obtained when the temperature for the initial interaction of the virus with the cell is lowered from 37° to 4° C

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Martin & Work (5) obtained a steeper growth curve of LMC virus in mouse ascites cells by rapid elevation of the temperature of the cell virus complex from 4° to 37° C This effect was explained as being due to a thermal shock resulting in a fair degree of synchroneous virus growth A similar mechanism may be partly responsible for some of the increased virus yield recorded in our experiments A thermal shock can, however, hardly account for the increased yield by attachment at 22° C compared with that obtained when the attachment took place at 37° C

Crawford (2) found no increase in the plaque titres after adsorption at low temperatures Further experiments are being performed to test if the advantage of the low temperature interaction reported in our experiments concerning haemagglutinin production can also be applied to plaque titrations

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THE BLOOD GROUPING AND THE BIOCHEMISTRY LABORATORIES OF THE STATE SERUM INSTITUTE, HELSINKI, FINLAND

DOUBLE DIFFUSION PRECIPITATION AND IMMUNOELECTROPHORESIS OF E. COLI B ANTIGENS

By

AITO LAHTI, JUSSI SAUKKONEN, TAPANI VAINIO
and YRJÖ ALLAS

Received 13 iii 63

Antigens of several bacterial strains have been studied with the aid of double diffusion precipitation (DDP) and immunoelectrophoresis (IE). A thorough investigation of mycobacterial antigens has been conducted by *Lind* (1961). He demonstrated by means of the DDP technique the presence of 17 different precipitinogens in a mycobacterial strain and investigated their occurrence in others. Certain physical chemical and immunological characteristics of these antigens were also reported. *Hanson & Holm* (1961) found 12 distinct antigens in the IE of streptococcal antigens, identifying among them Dick's toxin, streptokinase and streptolysin. *Fellon et al.* (1958) found a large number of antigens in the IE of ultrasonically disrupted preparations of H. pertussis. They suggested that some of their antigens might be products of enzymatic degradation of the native antigens. Furthermore *Grabar* (1960) found 9 precipitinogens in Salmonella while investigating O- and Vi antigens.

It is evident that DDP and IE are excellent tools in the study of microbial antigens. We have applied these techniques to the analysis of antigens of Escherichia coli, which have been intensively investigated in the past with the aid of other immunological techniques. This paper describes some of the characteristics of the antigens of E. coli B as revealed by these techniques.

METHODS

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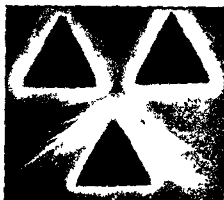


Fig 1

Precipitation patterns arising from the reaction between anti-E coli B serum and E coli B antigen material in DDP Bacterial antigen is in the two upper reservoirs and the antiserum in the lower reservoir



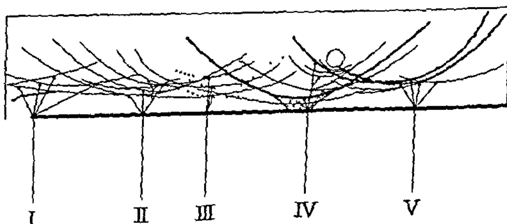


Fig 2

Immunoelectrophoresis of E coli B antigens For explanations see text

For experiments with DDP and IE the disrupted cells were centrifuged at 18000 × g for 60 minutes. The supernatant which contained about 5 per cent of protein was used for precipitation tests.

Immunization. Four rabbits were immunized with the antigen preparation. Each rabbit was inoculated subcutaneously with an initial dose of 5 ml of antigen preparation which had been diluted 1 : 1 with complete Freunds adjuvant. The immunization was continued with 2 ml of antigen once a week intramuscularly. The increase in antibody titer was checked monthly. After 3 months the animals were sacrificed and the blood collected by cardiac puncture.

Immunological tests. Both macro- and microtechniques of the DDP were used (*Ouchterlony* 1958). When the macromethod was employed the basins were refilled 3–4 times before the incubation was started at room temperature.

The method for micro IE was that of *Scheidegger* (1955). It was found convenient to carry out comparative IE (*Wadsworth & Hanson* 1960) on the same plate as the conventional IE.

The precipitation patterns were recorded photographically, after which the plates were dried and stained with Lissamine green.

RESULTS

The DDP and IE patterns of E. coli B antigens with rabbit anti E. coli B sera are seen in Figs. 1 and 2. In DDP about 12–13 bands are seen. The majority of the precipitation bands were formed close to the antibody reservoir. In IE up to 24 bands could be demonstrated. We have arranged the bands in five main groups as shown in Fig. 2. The first gro[illegible] n bands. It is [illegible] third group [illegible] migrates only slightly towards the anode and is composed of 6 bands. The fifth group, which consists of 4 bands, moved slightly towards the catode.

DISCUSSION

The good resolution of a large number of E. coli B antigens which was obtained with IE seems to invite other applications of the procedure. For instance, it would be interesting to compare the patterns of precipitation bands from several strains of E. coli, e.g. by utilizing the above mapping principle. Another logical extension of this investigation would be to try to correlate the antigens found in our studies with those revealed by other techniques.

In the present study no attempts were made to further fractionate the antigenic material. Therefore o[illegible] e.g. the obser[illegible] their rabbit a[illegible] with various [illegible] heterologous RNA's. More detailed analysis of the antigens described in this paper could be pursued along the lines described by *Bussard* (1955).

SUMMARY

E. coli B antigens have been studied with the aid of double diffusion precipitation and immunoelectrophoresis. The former technique reveals

12 13 antigens whereas 24 antigens can be observed by the latter procedure The antigens found in IE have been divided into five groups

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FROM STATENS SERUMINSTITUT, AMAGER BOULEVARD 80, COPENHAGEN S
STREPTOCOCCAL DEPARTMENT (CHIEF: KNUD SKADHAUGE, M.D.)

AUTOCLAVING OF SYRINGES IN PRESSURE COOKERS[1]

By

EBBE AHRENSBURG CHRISTENSEN, KNUD SKADHAUGE and
ANDREE RAVN-JONSEN

Received 18.i.63

Syringes may be sterilized by dry heat or by moist heat. The former procedure is most often recommended for hospital use (1, 2). Among the advantages of the method are that the syringes can be sterilized assembled and in sealed packages, and the question of subsequent drying does not arise. Its disadvantages—particularly with regard to its use in general medical and nursing practice—are that the actual apparatus is relatively expensive, and the method can fail, because it is often difficult to judge when the temperature in the load has reached the required value. In addition, anything less than scrupulous cleaning of syringe and needle involves the risk of retaining fragments of organic matter which may burn together with the internal surfaces, thus compromising the effectiveness of subsequent mechanical cleaning. Finally, dry sterilization is a relatively protracted process.

Bacterial spores are killed most rapidly and effectively by using saturated steam at a temperature of 120° C and upwards. In recognition of this fact, autoclaving by means of an ordinary domestic pressure cooker has in the course of time been repeatedly advocated for general medical practice.

McCulloch (3) emphasizes the following advantages of this simple type of autoclave: an excess pressure of 1 atmosphere is reached very rapidly, corresponding to a saturated steam temperature of about 120° C; the relatively small volume of the container permits removal of the atmospheric air quite effectively by means of steam discharge for a few minutes before closing the pressure valve, the sterilized material can be removed when still so hot that there is no drying problem. He concludes by saying: "While not to be considered as approximating the safety of sterilization in a properly designed steam pressure sterilizer, these cookers offer more positive protection than does boiling." *Baumann* (4) likewise recognizes the practicability of a simple pressure cooker and finds that its greatest disadvantage is that the load cannot

[1] Part of this paper was published in Ugeskr. Læg. 124: 1131, 1962.

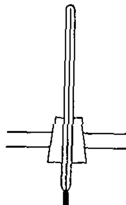


Fig 1

The lid of the pressure cooker with the thermometer The excess pressure in the coooker squeezes the rubber stopper against the thermometer and the hole in the lid

be removed in sterile packing, ready for use *Jorgensen* (5) states that during autoclaving, syringes should be placed disassembled in open containers which are not closed until after sterilization, the effective duration of which should be 15 20 minutes

The present authors have been unable to find any systematic study of the efficiency of the sterilizing process when syringes and needles are autoclaved in a household pressure cooker

EXPERIMENTS

A domestic pressure cooker[2] with a volume of 6 litres was used in the experiments To permit measurements of the temperature in the cooker's free space and in the actual load, a hole was bored in the lid to fit a rubber stopper carrying a mercury thermometer (Fig 1) and thermocouple leads for a thermoelectric meter The load was placed on a rack in the cooker, so that it was not exposed to direct contact with the water in the bottom of the cooker

Dried spores of *B subtilis* (the strain used in spore preparations from Statens Seruminstitut for routine control of autoclaves, dry sterilizers and formalin ovens) were used to determine the efficiency of the sterilizing process The spore material was used in two forms as test preparations, *i e* a mixture of spores with sterile sand, dried under vacuum and packed in small double-walled paper bags, or as a suspension of spores in broth to give a standard spore density The latter was used to contaminate syringes and needles

During the process of autoclaving, the temperature in the pressure cooker was checked by the thermoelectric meter mentioned, so that a continuous record could be made of the temperature, both in the free space of the cooker and in the load

2 A Danish made pressure cooker 'Trumf'

a) *Experiments with Spore Preparations and Different Obstructions to the Steam*

Test preparations containing 1.5×10^9 dried spores of *B. subtilis* were placed in pairs in 6 test tubes (15 cm long, 2 cm diameter), which were closed in two different ways:

1) with non absorbent cotton wool only ("cotton wool stopper");
2) with non absorbent cotton wool + gauze and a further cover consisting of one layer of tissue paper and one layer of brown wrapping paper ("cotton wool gauze-paper stopper").

The tubes were placed in wire baskets on the rack in the pressure cooker which contained 500 ml of water and autoclaved at 120° C. After autoclaving the spores were inoculated into broth, incubated at 37° C and examined daily for growth of *B. subtilis*.

TABLE 1

Sterilization Experiments. Trials with Different Obstructions to the Steam

Trial no.	No. of spore preparations per trial	Cotton wool stopper autoclaving for		Cotton wool-gauze paper stopper autoclaving for			
		5 min	10 min	5 min		10 min	
		growth after 30 days	growth after 30 days	growth after		growth after	
				30 days	60 days	30 days	60 days
1	2 × 6	0/12*	0/12*	0/12*	0/12*	0/12*	0/12*
2	2 × 6	0/12	0/12	0/12	0/12	0/12	0/12
3	2 × 6	0/12	0/12	3/12	5/12	0/12	0/12
4	2 × 6	0/12	0/12	7/12	8/12	0/12	0/12
5	2 × 6	0/12	0/12	1/12	1/12	0/12	0/12
6	2 × 6	4/12	0/12	5/12	7/12	0/12	0/12
7	2 × 6	2/12	0/12	4/12	5/12	0/12	2/12
8	2 × 6	3/12	0/12	2/12	2/12	0/12	0/12
9	2 × 6	2/12	0/12	2/12	2/12	0/12	0/12
10	2 × 6	0/12	0/12	2/12	2/12	0/12	0/12
Total		11/120	0/120	26/120	32/120	0/120	2/120

* Numerator: Showing growth on reading.
Denominator: Number of spore preparations examined.

Table 1 shows the results of these experiments. No growth was observed after 30 days in the broth seeded with the test preparations from the tubes with *simple* cotton wool stopper and autoclaved for 10 minutes. When the period of autoclaving was only 5 minutes, growth was obtained from a total of 11 out of 120 spore preparations.

In trials with the greater obstruction to the steam, *i.e.* cotton wool-gauze paper stopper, autoclaving for 5 minutes resulted in growth in 26 out of the 120 spore preparations when the cultures were followed for 30 days. On extending the period of observation to 60 days, the number of preparations showing growth increased to 32 out of the 120

Following autoclaving for 10 minutes none of the spore preparations (0/120) gave rise to growth within 30 days, while growth appeared in two out of 120 when the observation period was extended to 60 days

b) *Experiments with Spore-Contaminated Syringes and Needles*

Syringes and needles were contaminated with approximately 1 × 10⁶ spores by drawing into the syringe unit (syringe + needle) an adequate amount of a suspension of *B subtilis* spores in broth which was kept at 2 to 5° C

The syringes were then disassembled, although the needle was left on the barrel, and the separate parts were left at room temperature for about 18 hours to allow the spore material to dry In experimental series no IV, drying was done at the same temperature, but under vacuum

A total of four experimental series were carried out

I With unpacked syringes and needles, placed in an open Petri dish during autoclaving

II, III and IV In these series, syringe units were wrapped in two layers of paper, the inner a layer of coarse filter paper and the outer a layer of brown wrapping paper, fastened with a string Prior to the packing, the needles were placed in tubes plugged with cotton wool

A total of 145 syringe units were autoclaved at 120° C, grouped into four series with 25, 100, 15 and 5, respectively In series I and II the autoclaving time was 5 minutes, in series III and IV it was 10 minutes To check that *B subtilis* was not present in the vegetative form but in form of spores, 30 syringe units were heated in the pressure cooker at 100° C (25 for 5 minutes, 5 for 10 minutes) Finally, a number of unheated control units were included in each experimental series to determine the degree of contamination (Experimental series nos III and IV were run together, so that the 5 control units indicated in Table 2 under experimental series no III were common to both series III and IV Altogether a total of 40 control units was examined)

The number of surviving spores per syringe unit was determined by rinsing the syringe and needle with 10 ml of chilled broth Ten-fold dilution series were prepared from the rinsings, and 0 1 ml portions of these were plated on blood agar for colony counting Both blood agar plates and the remainder of the original rinsing fluid (broth) were incubated at 37° C The broth was observed for growth for a period up to 30 days

Table 2 shows the results of the experiments In none of the trials was there any growth of *B subtilis* on plating from the rinsings from the syringe units which had been autoclaved at 120° C Nor did the actual broth for rinsing show growth on observation for up to 30 days The insignificant difference between the counts obtained on plating from the control syringe units and from the units which had been heated at 100° C indicated that the bacteria had been in the spore stage during the trials

TABLE 2

Sterilization Experiments Trials with Spore Contaminated Syringes and Needles

Series	Wrapping of syringes	Heat treatment	Heating minutes	No of syringes	No of spores per unit*	Remarks
I	unwrapped	approx 120° C	5	5 × 5	0	The contaminated syringes dried overnight at room temperature
		100° C	5	1 × 5	2 5 × 10^6	
		untreated control		3 × 5	3 8 × 10^6	
II	wrapped	approx 120° C	5	20 × 5	0	do
		100° C	5	4 × 5	2 3 × 10^6	
		untreated control		4 × 5	4 5 × 10^6	
III	wrapped	approx 120° C	10	3 × 5	0	do
		100° C	10	1 × 5	2 5 × 10^3	
		untreated control		1 × 5	4 0 × 10^6	
IV	wrapped	approx 120° C	10	1 × 5	0	The contaminated syringes dried overnight under vacuum

* Mean count of spores per syringe unit (syringe + needle)

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		untreated control		3 × 5	3.8×10^6	
II	wrapped	approx 120° C	5	20 × 5	0	do
		100° C	5	4 × 5	2.3×10^6	
		untreated control		4 × 5	4.5×10^6	
III	wrapped	approx 120° C	10	3 × 5	0	do
		100° C	10	1 × 5	2.5×10^6	
		untreated control		1 × 5	4.0×10^6	
IV	wrapped	approx 120° C	10	1 × 5	0	The contaminated syringes dried overnight under vacuum

* Mean count of spores per syringe unit (syringe + needle)

Temperature Measurements

During the experiments, the temperature in the free space of the pressure cooker was measured both by mercury thermometer and by thermocouple. The temperature within the load was measured by thermocouple.

In all cases, there was a satisfactory agreement between temperatures, that of the load reaching the same maximum value as that of the free space, the greatest delay in doing so being 1 *minute*.

DISCUSSION

"Sterilization" of syringes and needles is in many cases performed by boiling. However, this method of disinfection can be considered justified only if the mechanical cleaning prior to boiling and the entire procedure for handling the syringes and needles are absolutely irreproachable.

As mentioned, autoclaving by pressure cooker has been proposed repeatedly as a solution to the problem of sterilization in general practice. The fact that the method has not found wider use is presumably due to an uncertainty as to its effectiveness, and also to an impression that it suffered from the disadvantage that the sterilized material could not be removed in a packing suitable for storage, and that subsequent drying was necessary. The classic form of packing in two layers of paper is well suited to autoclaving, and the experiments described here show that this packing can be used in a pressure cooker. Evacuation of the atmospheric air merely by blowing off steam before attaching the pressure valve is surprisingly effective.

Autoclaving of syringes + needles contaminated with approximately 10[illegible] spores resulted in complete destruction of the spores in five minutes. Assuming that the destruction of the bacteria is an exponential function of time, the above result implies an exceedingly great safety factor when a routine autoclaving period of 20 minutes is employed, since such an increase in the sterilization time will give theoretically a reduction factor of more than 10^{20}.

The present experiments using test preparations of spores show that a small increase in the obstruction to the steam will result in a reduction in the efficiency of the sterilizing process.

The possibility cannot be excluded that barriers to the steam greater than those employed in the present experiments might result in *failure* of the method, even with boiling under pressure for 20 minutes. It is considered advisable, therefore, that the type of pressure cooker available should be controlled for its efficiency of sterilization, and that the method of packing to be used should likewise be checked. Control will be necessary

1) if the barriers to the steam are judged to exceed those of the present experiments,

2) if there is any doubt that the temperature in the cooker has reached 120° C

Control can be maintained by placing 10 test preparations in the sites most inaccessible to steam and then boiling under pressure for *10 minutes* If all the preparations are found to be sterile a good safety margin can be reckoned with in *routine sterilization for 20 minutes*

All pressure cookers used for sterilization should be fitted with a thermometer The sterilization time should only be calculated from the moment the thermometer shows at least 120° C and sterilization should not be for less than 20 minutes In the present experiments the observation made by *McCulloch* was also confirmed namely that the sterilized material can be removed at a stage when its heat content is so great that in most cases subsequent drying becomes unnescessary

SUMMARY

An account is given of the efficiency of the sterilizing process in a household pressure cooker With an autoclaving period of only 5 minutes and using wrapped syringe units (syringe + needle) contaminated with approximately 10^6 dried spores of *B subtilis*, a reduction factor of about 10 was obtained

The method appears suitable as a routine for sterilizing syringes in general medical and nursing practice

It is concluded that a pressure cooker used for sterilization should always be fitted with a thermometer and a temperature of at least 120° C should be employed for 20 minutes

The possibility of a control of the method is discussed

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BRIEF REPORT

AFFINITIES BETWEEN MORAXELLA SPP AND A STRAIN OF NEISSERIA CATARRHALIS AS EXPRESSED BY TRANSFORMATION

By *Kjell Bøvre*

Catlin & Cunningham (1961) reported that Neisseria catarrhalis is distinct from other neisseriae in terms of DNA base *composition and transformation Marmur & Doty* (1962) reported similar base composition of DNA s from Moraxella bovis and Neisseria catarrhalis (strain Ne 11 Catlin)

Material and methods A strain of Neisseria catarrhalis (Ne 11 Catlin) was subjected to the action of crude DNA extracts from streptomycin resistant mutants of four strains of Moraxella nonliquefaciens and one strain of Moraxella bovis All these Moraxella strains had previously been found representative for their respective species as expressed by isospecific and heterospecific transformation ratios in quantitative experiments including 21 Moraxella nonliquefaciens strains and four Moraxella bovis type strains (*Bøvre*) In addition one type strain of Moraxella liquefaciens was included in the experiments as donor of streptomycin resistance

With slight modifications the procedure was that of Bøvre & Henriksen (1962) The controls included parallels with non marker DNA marker DNA treated with DNase before use and different extracts of the same donor strain As a negative control also served DNA extracted from a streptomycin resistant mutant of Neisseria meningitidis which had proved active in intraspecific transformation

Results The strain of Neisseria catarrhalis was transformed to streptomycin resistance by DNA s from all the strains of Moraxella nonliquefaciens and Moraxella bovis The transformation frequency was low but reproducible and distinct from the spontaneous mutation frequency in quantitative experiments with the ordinary 15 *minutes* DNA exposure The ratios of inter generic to homospecific transformation frequencies were of the order 1 4 10^{5} without significant difference between the effects of the two donor species (homospecific transformation frequencies *ranged from one to two per cent of the recipient population) When DNase treatment* after 15 minutes DNA exposure was omitted in additional non quantitative tests DNA was found to have an effect on the growing agar culture through the 6 7 hr phenotypic *expression period giving rise to several hundreds of transformant* colonies per plate as compared with 0 2 spontaneous mutant colonies per control plate

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the Neisseria catarrhalis recipient even when DNase treatment was omitted

Comment The above mentioned ratios of inter generic to homospecific transformation frequencies in the reactions *between Moraxella spp and Neisseria catarrhalis* may be compared with the ratios ? 7 10 3 of heterospecific to homospecific transformation to streptomycin resistance *between* strains of Moraxella nonliquefaciens and Moraxella bovis (*Bøvre*) *The importance of the low frequency inter generic reactions* reported is unknown An extended study on this subject is therefore indicated

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INDEX

VOL 58 FASC 1–4 1963

SUPPLEMENTA